THE UNIVERSITY OF ALBERTA

METABOLIC CHANGES OF SERUM ESTROGENS AND BILE ACIDS DURING PREGNANCY AND ORAL CONTRACEPTION

by

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A THESIS

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TO MY HUSBAND MIKE AND OUR CHILDREN DANIEL, CHRISTOPHER, JAN AND MARIA.

ABSTRACT

Pregnancy and the estrogenic components of oral contraceptives seem to play a part in the susceptibility of women to cholestasis and cholelithiasis. Etiology of the increased bile lithogenicity may result from a reduction in bile synthetic and secretion rates, reduction of pool size, and an increase in cholesterol saturation. Increased levels of estrogens, common to both pregnancy and oral contraception, were postulated to play a causative role in the epidemiology of cholestasis and gallstones. It was felt that there might be a relationship between estrogen catabolism and bile acid synthesis, whereby a common hydroxylating enzyme pathway for bile acids and estrogens may exist.

The effects of normal pregnancy, cholestasis of pregnancy, and oral contraception on bile acid and estrogen metabolism were studied. Total serum bile acids were measured by a modified enzymatic-fluorimetric technique, and the unconjugated estrogens, estrone, estradiol, and estriol, by independent radioimmunoassay procedures. Standard liver function tests were also performed.

Normal pregnancy caused a significant increase of total serum bile acid levels which dropped slowly in the pueperium. No significant differences could be found between non-pregnant controls and women on oral contraceptives. Total bile acids were markedly increased in cholestasis of

pregnancy, but not in pruritus gravidarum. Unconjugated estrogens increased during pregnancy at a faster rate than the bile acids, whilst levels in cholestasis were significantly higher than those seen in normal pregnancy. Despite marked increases of bile acids and total estrogens, ratios of total bile acids to total estrogens resembled those of normal pregnancy. The latter showed estradiol as the most prominent estrogen, however, this pattern reversed in cholestasis of pregnancy, where estrone was dominant and estradiol tended to be lower than normal. Relative amounts of each of the estrogens were constant throughout normal pregnancy with a disruption of this balance in cholestasis. Estrone levels were abnormal in pregnant women with symptoms of pruritus without clinical evidence of cholestasis.

It was concluded that a tendency towards cholestasis occurs in normal pregnancy, reflected by increasing levels of unconjugated estrogens and bile acids, caused by metabolic changes and gradual reduction in hepatocyte secretion of these steroids. An increasing estrogen load from the developing fetus is accompanied by an increasing inefficiency of estrogen catabolism in the mother, this tendency exemplified in cholestasis of pregnancy where deficient hepatocytic hydroxylation or conjugation of estrone is indicated. Estrogens, particularly estrone and estradiol, probably affect liver microsomal enzyme systems, resulting in deficient hydroxylation and conjugation of estrogens and bile acids. This may lead to decreased

micellar formation and hepatocyte secretion, with subsequent build-up of hepatotoxic monohydroxy or dihydroxy bile acids and the initiation of cholestasis. After parturition when estrogens are no longer present to effect a disturbance in hepatocyte function, bile acid bydroxylation and secretion gradually return to normal and symptoms of cholestasis disappear.

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INTRODUCTION

Gallstones and gallbladder disease have, in the past, been associated with: "fat, fertile females of forty",[1] thus incriminating diet, parity, sex and age in the etiology of cholelithiasis.[2 3 4] The incidence increases with age in both sexes, but is twice as common in women under 45 years of age as in men,[5 6 7] being greater in multiparous than in nulliparous women.[8 9] This has also been demonstrated in an animal model.[10]

In recent years, the increased incidence of gallstones and cholestasis in younger women may demonstrate more than a coincidence with the fact that many young females have commenced using oral contraceptives within the past 15 years.[11 12] Although diet, environment and genetic factors may be involved,[13 14 15 16 17] mounting evidence indicates that pregnancy and female sex hormones are important factors in the increased susceptibility of females to cholestasis and gallstones. Nilsson in Sweden,[18] found a similar prepubertal incidence of cholelithiasis in both sexes, with an increased female preponderance at puberty. Women using oral contraceptives and post-menopausal women on estrogen therapy, have a 2.0 to 2.5 times greater risk of developing cholelithiasis and cholestasis than women not taking these hormones.[19 20] Bile from women on oral contraceptives was recently shown to be supersaturated with cholesterol, reverting back to non-lithogenicity after they stopped

taking these hormone perparations.[21] At least 8 million American women are taking oral contraceptives; therefore, one can estimate at least 10,000 new cases of cholelithiasis each year in the United States alone, as a result of oral contraception.[22] The increased incidence of cholelithiasis with parity, has been recently related to reduced hepatic synthesis and secretion of bile acids as well as pool size, during normal pregnancy in rats.[23]

Cholestatic jaundice of pregnancy is of unknown etiology; however, this apparently occurs most frequently in women who have used oral contraceptives prior to becoming pregnant.[24] Since increased levels of estrogens are seen in pregnancy and following administration of "estrogen-containing contraceptives", these hormones are suspected of having a role in the development of cholestasis and gallstones, particularly in multiparae, or following continuous contraceptive therapy. The frequency of cholestasis is greatest in late pregnancy when estrogen levels are highest.[25]

We felt there might be a relationship between estrogen catabolism and bile acid synthesis in the liver, and considered the possibility that the cytochrome P450 system, which plays an important role in microsomal hydroxylation[26] and consequently the synthetic pathway for bile acids, may also be associated with estrogen hydroxylation. Thus, in women with high estrogen levels, there could be competition

between bile acids and estrogens for available enzyme, with a net decreased hydroxylation of these steroids. Marginal or decreased hepatocytic enzyme levels in susceptible women, would explain the tendency for these women to develop cholestatic jaundice when pregnant or on oral contraceptives.

OBJECTIVES

The objectives of this study were to:

1. develop laboratory procedures for determinations of total bile acids, the unconjugated estrogens (estrone, estradiol, estriol) in serum.

2. apply these methods to a clinical study of pregnant and non-pregnant women in order to:
   a) determine the effect of pregnancy and oral contraceptives on estrogen and bile acid metabolism.
   b) determine if there is a relationship between bile acids and the unconjugated estrogens and how this relationship is affected in normal pregnancy and cholestasis of pregnancy.
   c) postulate the possible role of estrogens and bile acids in initiating cholestasis during pregnancy or oral contraception.

3. measure changes of liver function which occur during normal pregnancy, cholestasis of pregnancy and oral contraception, as compared to normal non-pregnant controls.

CHAPTER I

REVIEW

A. Cholestasis

Definition and Characteristics

Cholestasis, according to Popper,[27] is a "disturbance in the secretion of micelles of bile salts mixed with other solids," resulting in a reduced output of bile salts into the bile and intestine.[28] Physiologists regard cholestasis as the interference with bile flow somewhere between the hepatocyte and intestinal tract.[29]

Extrahepatic cholestasis occurs between the bifurcation of the common hepatic duct and ampulla of Vater due to obstruction, with resultant hydrohepatosis and dilatation of proximal biliary passages. Intrahepatic cholestasis may be due to chemical or structural changes in the hepatocyte secretory mechanism, bile constituents, or transport membranes. Regurgitation of bile into the blood and lymph occurs in both types of cholestasis.[27] [28] Pathologists recognize cholestasis by the morphological changes of canalicular dilatation, bile pigmentation of hepatocytes and Kupffer cells, inspissated bile plugs, and subsequent feathery degeneration with hepatocyte necrosis.[30] [31]

Clinicians regard cholestasis as jaundice, associated with diminution or absence of bile flow, pruritus,

hepatomegaly, xanthoma, easy bruising, grey colored stools, and dark colored urines.[28] Secondary biochemical changes include conjugated hyperbilirubinemia with high serum alkaline phosphatase, aminotransferases, 5'-nucleotidase, cholesterol and lipoprotein levels.[32 33 34] The abnormal serum lipoprotein-X is found in extrahepatic cholestasis.[35] Serum bile acids are increased and probably cause pruritus when deposited in the skin.[36 37] Cholic acid, normally the predominant bile acid in the liver, rises conspicuously with severe cholestasis, and the sum of the detergent-acting dihydroxy bile acids, chenodeoxycholic and deoxycholic acids, parallels the degree of feathery degeneration and hepatocyte necrosis.[38 39]

Mechanisms of Cholestasis

Scaffner[40] recently summarized some possible mechanisms of cholestasis. They include:

1. abnormal bile salt concentrations altering membranes by detergent action,
2. interference with micelle formation by drugs or monohydroxy bile acids,
3. inhibition of non-bile salt dependent bile flow,
4. alteration of bile canalicular membranes,
5. increased intraluminal pressure in the biliary system due to obstruction.

The primary event in intrahepatic cholestasis is alteration of hepatocytic smooth endoplasmic reticulum, leading to disturbed bile acid hydroxylation which in turn interferes with micellar formation, resulting in disturbed secretion of bile salts.[27 36 41] Secondary events which follow are: additional damage to the smooth endoplasmic reticulum and a further reduction in enzyme activity.

The monohydroxy bile acid lithocholate, a potent hepatotoxin, is endogenously synthesized by colonic bacterial degradation of chenodeoxycholate, and normally excreted in the feces,[29] however it has been shown to induce cholestasis in animals.[42 43] This led to the hypothesis that lithocholate or some monohydroxy bile acid altered hepatocytic micellar formation.[27 33] Rats with bile duct ligations and established cholestasis, had markedly reduced cytochrome P450 activity in their hepatocytes.[44] Defective P450 dependent 7α-hydroxylation of cholesterol,[45] in hypertrophic, hypoactive endoplasmic reticulum, is associated with inability of the hepatocyte to synthesize cholate or chenodeoxycholate, with consequent production of monohydroxy bile acids.[33 41] Sensitivity to lithocholate shows species variation,[46] and although significant quantities of lithocholate have not been reported in man with severe cholestasis,[39] conjugated, non-toxic sulfates of lithocholate have been demonstrated in human bile[47] and urine.[48]

Increased quantities of chenodeoxycholate and deoxycholate can produce hepatotoxicity,[49] [50] and by their detergent action, inactivate cytochrome P450 the terminal oxidase of microsomal enzyme systems, forming inactive P420, as well as solubilizing other microsomal membrane components.[51] Rats with cholestasis do not demonstrate high chenodeoxycholate levels, because of an active hepatic 6β-hydroxylase, which catalyzes the formation of non-toxic β-muricholic acid. Hepatocellular necrosis is rarely seen in the rat after bile duct ligation.[27] [40] In man, however, this hydroxylation does not occur, and high levels of chenodeoxycholate seen with long-standing cholestasis, may account for pruritus and hepatocellular necrosis.[27] [52] In short-term cholestasis, sulfation and glucuronidation in the smooth endoplasmic reticulum may prevent accummulation of this bile acid.[53] [54]

Steroid molecules with a phenolic A ring or 17 alkyl substitution appear to be cholestatic, but no precise mechanism based on chemical configuration, has been determined.[55] It has been suggested that estrogens induce cholestasis by increasing permeability of the biliary tree, and enhancing diffusion of materials from the bile into the blood, as well as inhibiting active transport in the opposite direction.[56]

Since cholestasis is related to a disturbance of bile acid metabolism and decreased hepatocyte secretion, the

consequent production of a lithogenic bile would predispose to cholelithiasis.[57]

B. Cholestasis and Pregnancy

History

Jaundice of pregnancy with a recurrent tendency in subsequent pregnancies, was first described in 1883 by Ahlfeldt,[58] and was further elucidated as a cholestatic disturbance in the nineteen fifties by Svanborg[59 60] and Thorling.[61] Many synonymous terms have been used to describe this hepatic disorder. They include: hepatosis of pregnancy,[25] idiopathic cholestasis of pregnancy,[62] recurrent jaundice of pregnancy,[59] obstetric cholestasis,[63] and others.[64] Pruritus gravidarum is a milder form of the disease.[65 66]

Clinical Aspects

The incidence of cholestasis of pregnancy has been found to vary from 0.02 - 2.4 percent of all pregnancies, in Scandanavia and Chile.[61 67 68] A familial occurrence of the disease has been reported,[68] and some authors have suggested a hereditary predisposition to cholestasis of pregnancy.[69 70]

Symptoms usually occur during the last trimester of pregnancy[27] subsiding after parturition, with recurrence in

subsequent pregnancies.[71 72 73] The initial and dominant symptom in cholestasis of pregnancy is pruritus, which may or may not be followed by slight jaundice and cholestasis.[27] A history of vague anorexia, nausea, vomiting, epigastric pain and diarrhea, are less common findings.[58 60 61]

A tendency towards a prolonged prothrombin time [58 65] and hemorrhage [60 61] at parturition has been reported in some cases. There tends to be a significant fetal mortality rate,[74] related to an increased occurrence of premature infants of low birthweight,[75 76 77] and a short duration of labor with intense uterine contractions.[78]

A high incidence of gallstones (40%) has been reported among Scandanavian women who have a previous history of cholestasis during pregnancy or while taking oral contraceptives.[66]

Morphology

Liver biopsies from patients with "cholestasis" of pregnancy demonstrate histological changes similar to those seen in other cholestatic disturbances.[58 61 79 80] There is little or no inflammation of portal connective tissue, and parenchymal cell necrosis is minimal or absent.[27 58 61 65]

Laboratory Findings

Serum bilirubin levels in normal pregnancy usually

remain within normal limits,[64] however in cholestasis of pregnancy, slight elevations occur, but rarely exceed 5mg/dl.[69] Johnson[81] categorized pruritic patients with serum bilirubin levels greater than 1.2mg/dl, and/or aminotransferase levels greater than 2 1/2 times the normal, as cholestasis of pregnancy, classifying lesser changes as pruritus gravidarum.

Alkaline phosphatase activity is increased in normal pregnancy,[61] [82] due to the placental isoenzyme.[83] Cholestasis of pregnancy, leads to a further increase of alkaline phosphatase activity, with a slow return to normal following parturition.[58] [61] The aminotransferases[62] [84] and serum 5'-nucleotidase usually remain within normal limits during pregnancy, but are moderately increased in cholestasis of pregnancy.[85] [86]

Bromsulphalein (BSP) excretion is impaired and its relative storage capacity increased during the last half of pregnancy.[87] Both changes are reversible after parturition.[88] These findings led others to state that "pregnancy in itself is cholestatic".[89] A further increase in retention of conjugated BSP-glucuronide is seen in cholestasis of pregnancy and a disorder in enzyme activity affecting active transport through the liver cell membrane, is thought to be present.[27] [90]

Serum lipids increase throughout normal pregnancy, cholesterol maxima being reached during the third

trimester.[64 91 92] These changes are considered to be hormonally induced.[62 66] Greater increases of serum lipids, cholesterol, and abnormalities in lipoproteins, have been reported in cholestasis of pregnancy,[92] with a dramatic increase in serum bile acid concentrations, levels approaching 10 to 100 times those seen in normal pregnancy and the non-pregnant state.[93 94] The ratios of cholic, chenodeoxycholic, and deoxycholic acids, are grossly disturbed in cholestasis of pregnancy as compared with normal pregnancy, showing marked increases in cholic and chenodeoxycholic acid concentrations.[93]

Plasma and urinary estrogens also show changes from normal pregnancy,[95] but, these changes have been considered to be secondary to impaired enterohepatic circulation of estrogens.

Treatment of Cholestasis of Pregnancy

The use of cholestyramine as a bile acid sequestering agent has been recommended to reduce systemically circulating bile acid concentrations. Remaining therapuetic measures are mainly palliative (diet and rest).[80 96]

Pathogenesis

The etiology of cholestasis of pregnancy has not been resolved, but evidence in support of a hormonal cause[80 69] has been documented following the changes seen in patients

taking oral contraceptives.[86 97 98] Estrogen production is not increased in cholestasis of pregnancy,[99] but compared to normal pregnancy, decreased urinary estriol conjugates have been found, which may be related to a primary reduction in biliary secretion of estrogens.[95] The possibility of an inherent enzyme defect leading to an increased sensitivity and failure to metabolize the estrogens normally present in pregnancy, has been suggested as a causative factor.[76]

C. Oral Contraceptives and Cholestasis

The jaundice and cholestasis which occasionally occur in women receiving combined oral contraceptives, usually appear within one month, and may be preceded by anorexia, nausea, malaise, and pruritus.[100] Morphological and biochemical changes are remarkably similar to those of cholestasis of pregnancy.[62 66] A large percentage of these individuals have a history of jaundice and/or pruritus,[62] and discontinuation of the therapy leads to a complete remission of symptoms within a few weeks.

CHAPTER II

BIOSYNTHESIS AND METABOLISM

A. Cholesterol

Source

Cholesterol is utilized in cell membrane structure, and in the synthesis of bile acids and steroid hormones. It is derived from dietary (0.5g/day) and endogenous (1-2g/day) sources, the latter synthesized from acetyl CoA, resulting from the metabolism of carbohydrate, fat, and protein.[101] Production occurs primarily in the liver and intestinal mucosa. The liver pool of cholesterol has an enterohepatic circulation, whereby it is excreted in the bile into the duodenum, mixing with dietary cholesterol and partially reabsorbed with bile salts and phospholipids, the fecal excretion varying from 1g to 3g/day.[102] Dietary cholesterol, following esterification and absorption in the intestinal mucosa, is hydrolyzed in the liver, with incorporation into the hepatic pool of unesterified cholesterol.[64]

Biosynthesis

Hydrolysis of cholesterol esters in the liver cell occurs in the "cell sap", catalyzed by cholesterol esterase, whereas esterification occurs in the microsomes and mitochondria. Three molecules of acetyl CoA, Figure 1,

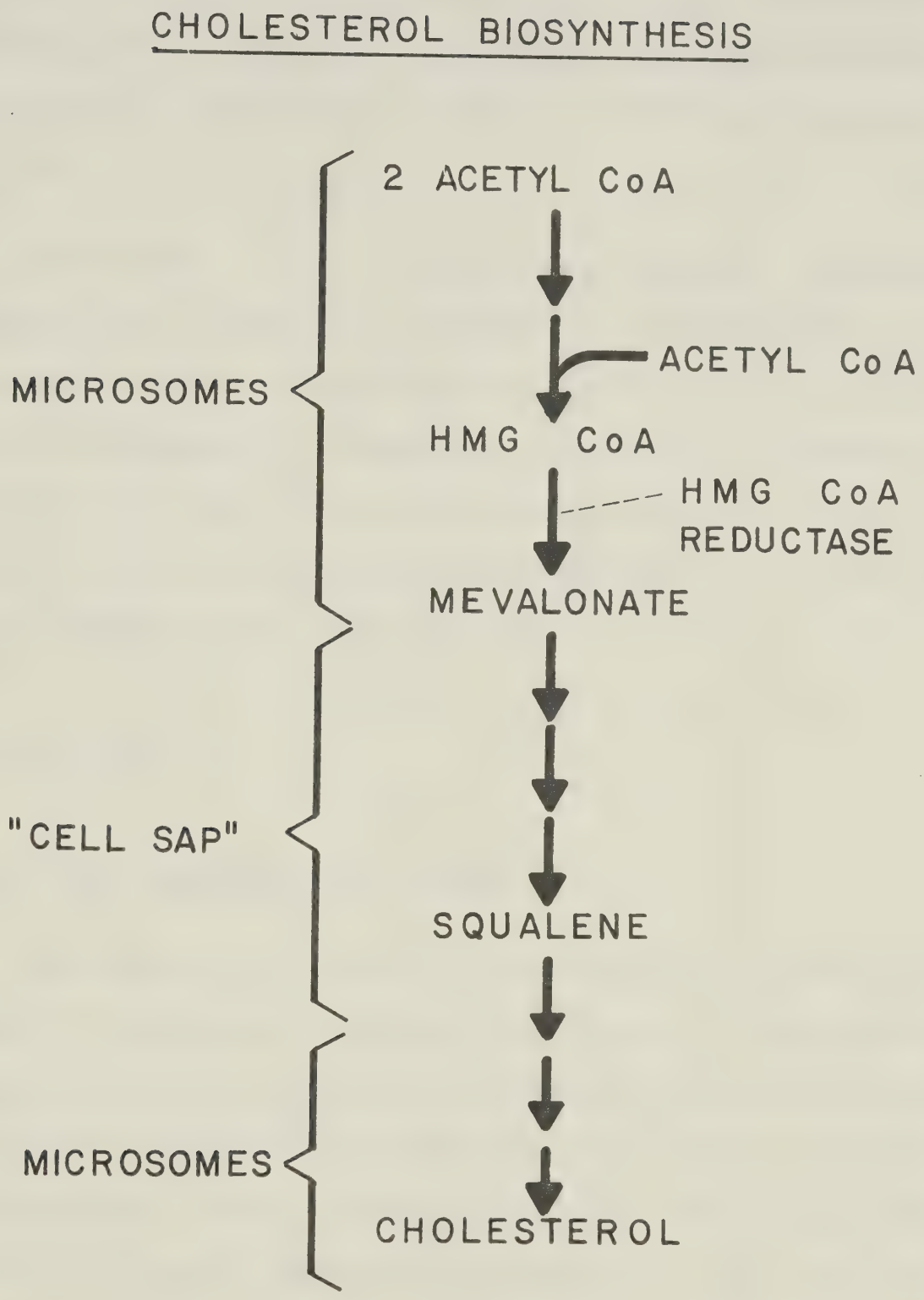


Figure 1. Cholesterol Biosynthesis in the Liver Cell.

condense to form β-hydroxy-β-methyl-glutaryl CoA, (HMG-CoA) which is then reduced to mevalonic acid by microsomal HMG-CoA-reductase, the rate controlling step of cholesterol synthesis.[101] Hormonal and bile salt derivatives are postulated as controlling factors of HMG-CoA-reductase.[103] [104] [105]

Mevalonate is converted through intermediate phosphorylated compounds to squalene, which through a series of redox reactions is cyclized to lanosterol. Cholesterol is finally synthesized by changes in the A ring structure and reduction of the side chain double bond. A portion of the newly synthesized cholesterol is converted into bile acids before secretion into the bile.[104]

B. Bile Acids

Primary and Secondary Bile Acids

Cholesterol is reduced to cholanoic acid which is the immediate precursor of primary bile acids.[106] This synthesis occurs in the liver and produces cholic and chenodeoxycholic acids. The latter are secreted into the bile, stored and concentrated in the gallblader for later evacuation into the intestine, where bacterial degradation and reabsorption occur by means of the enterohepatic circulation. This circulation is extremely efficient in that a small pool of 2g to 4g of bile acids is recycled six to ten times each day

with a fecal loss of approximately 0.6g, which is replenished through normal hepatic synthesis. The formation and elimination of bile acids in the feces, is the main mechanism for the maintenance of normal cholesterol homeostasis.[107]

Secondary bile acids, formed in the intestinal lumen by anaerobic bacterial degradation of primary bile acids, results in the 7α-dehydroxylation of cholic and chenodeoxycholic acids forming deoxycholic and lithocholic acids respectively,[108] Figure 2. Normally 90% of biliary bile acids are conjugated, through a stable C24 amide bond, with glycine or taurine, in a molar ratio of 3 to 1, this is said to be species specific but may be easily modified by nutritional and hormonal factors.[109 110]

Biosynthesis and Conjugation

Biosynthesis of primary bile acids from cholesterol includes stereospecific reduction of the Δ^5 double bond, epimerization of the 3-hydroxyl group, introduction of hydroxyl groups at the 7α and 12α positions, with oxidation and reduction of the terminal three carbon side chain. This is the main pathway of bile acid synthesis,[111 112] but proposed alternate pathways may exist.[113]

(i) Cholic Acid

The first step in the synthesis of cholic acid, the

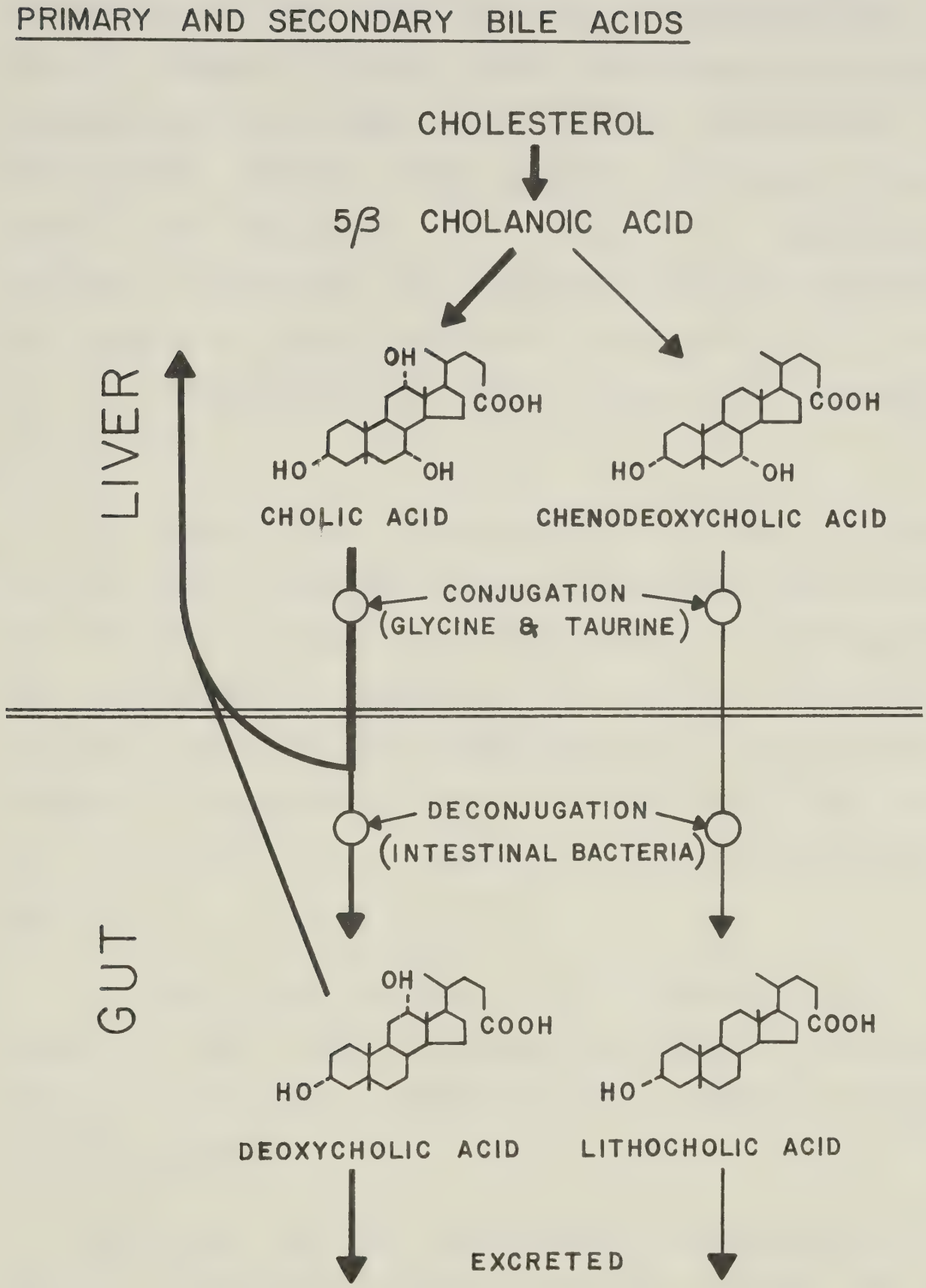


Figure 2. Primary and Secondary Bile Acids and the Enterohepatic Circulation

major primary bile acid in man and rat, is the 7α-hydroxylation of cholesterol to form 5-cholestene-3β, 7α-diol,[114] Figure 3. This highly stereospecific reaction, catalyzed by a microsomal enzyme complex (cholesterol 7α-hydroxylase), requires NADPH (reduced nicotinamide adenine dinucleotide phosphate) and oxygen.[115] Cytochrome-c reductase and possibly cytochrome P450 are also involved in the reaction,[116] which appears to be the rate-limiting step in the biosynthesis of both cholate and chenodeoxycholate.[117]

The second step, catalyzed by microsomal enzymes, requires NAD^+ (oxidized nicotinamide adenine dinucleotide) as a cofactor. Two enzymes, 3β-hydroxysteroid dehydrogenase and an oxosteroid isomerase, are presumed to act in a concerted manner.[118] The hydroxyl group of 5-cholestene-3β, 7α-diol is oxidized and the double bond shifts from the C5 to C4 position, with the formation of 7α-hydroxy-cholest-4-ene-3-one.

The next step involves C12 hydroxylation and the formation of 7α, 12α-dihydroxy-cholest-4-ene-3-one, catalyzed by microsomal enzymes requiring NADPH and possibly cytochrome P450.[119]

Conversion of 7α, 12α-dihydroxy-cholest-4-ene-3-one into 3α, 7α, 12α-trihydroxy-5β-cholestane, consists of two reactions catalyzed by non-microsomal oxosteroid-5β reductase and 3α-hydroxysteroid dehydrogenase in the

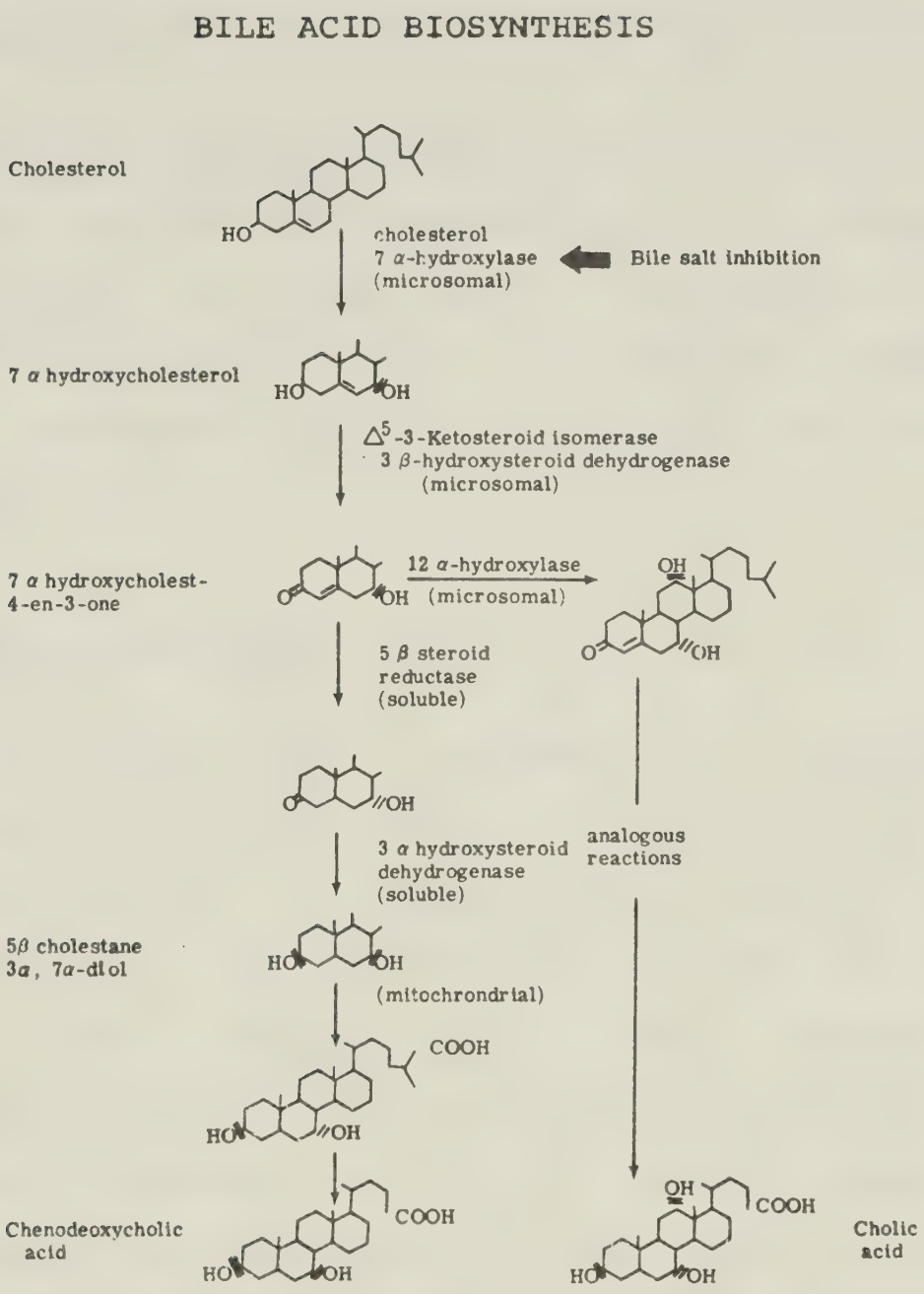


Figure 3. Main Pathways of Bile Acid Biosynthesis.

presence of NADPH.[120] Reduction of the 5C position completes a significant change in shape of the cholesterol molecule which is partly responsible for the detergent-like property of bile acids.[106]

Hydroxylation of 5β-cholestane-3α, 7α, 12α-triol at the C26 position, the first step in oxidation of the side chain, is sterospecific and occurs in the mitochrondria.[121 122] Further oxidation reactions lead to the formation of cholyl-CoA and the subsequent formation of glycine and taurine conjugates.[121 123]

(ii) Chenodeoxycholic Acid

Chenodeoxycholic acid synthesis from cholesterol, involves the same structural changes as in the formation of cholic acid; however, 12α hydroxylation does not occur. There is doubt as to where the synthetic pathways of these two bile acids diverge, and several pathways of chenodeoxycholate synthesis have been postulated.[109 113] 7α hydroxylation, oxidation of the 3β hydroxyl group, and isomerization of the Δ^5 double bond, are steps in common with the formation of cholic acid. Bjorkhem[122] has suggested that 7α-hydroxycholest-4-ene-3-one, the last intermediate common to both bile acids, will proceed to the formation of chenodeoxycholate if not immediately hydroxylated at the C12 position.

Interconversion of chenodeoxycholate to cholate does

not occur normally in man, although in biliary atresia, Palmer has recently shown a limited rate of conversion.[124] In rats, however, the synthesis of α and β muricholic acids is greatly enhanced by bile duct atresia. This is postulated as a hepatic mechanism in detoxifying chenodeoxy cholic acid, but is of little importance in man.[125] [126]

(iii) Conjugation

Conjugation of bile acids from their activated CoA intermediates in the soluble cytoplasmic fraction of liver microsomes,[127] is catalyzed by two distinct lysosomal acyltransferases,[128] for glycine and taurine.[129] Bile acids which are reabsorbed from the intestine after bacterial deconjugation, must be reconverted into their active CoA esters before reconjugation. This is catalyzed by a microsomal enzyme which requires CoA, Mg^{2+}, and ATP.[130]

Bile Acid Synthetic Control Mechanisms

Interruption of the enterohepatic circulation in man, results in a five to ten fold increase of hepatic bile acid synthesis,[123] [131] which led to the conclusion that there is a negative feedback control mechanism for bile acid biosynthesis.[132] [133] Enhanced cholesterol synthesis in the liver and intestinal mucosa raised the possibility that bile acids may also regulate cholesterol synthesis. Continuous biliary drainage and cholestyramine feeding result in

increased hepatocyte 7α-hydroxylase activity in rats, and the synthetic rate of cholesterol is also increased.[134] Bergstrom and others,[131 135] postulated that enhanced bile acid synthesis following total biliary drainage is caused by a reduction in the rate of bile acid return to the liver and that bile acids in portal blood inhibit 7α-hydroxylase activity in the hepatocyte. Supportive evidence for this theory was demonstrated by infusing pure bile acids into the duodenum of rats with bile fistulae and observing the accompanying reduction of bile acid synthesis.[133]

Schoenfield[136] showed that chenodeoxycholate and cholate administered to hamsters, inhibit HMG-CoA reductase and 7α-hydroxylase. He noted, however, that hepatic cholesterol and bile acid concentrations appeared to increase, postulating that this may be due to decreased release from the hepatic cell. Chenodeoxycholate inhibited cholesterol synthesis more than bile acid synthesis, whereas cholate inhibited bile acid synthesis more than cholesterol synthesis.

In man, chenodeoxycholate administration, to expand the content of the bile acid pool to contain greater than 90% chenodeoxycholate, demonstrates a suppression of cholate synthesis by approximately 50%, evidenced by marked reductions of cholate and deoxycholate pool sizes.[137] Cholate suppresses chenodeoxycholate synthesis by 50%,[138] and the administration of deoxycholate suppresses

chenodeoxycholate synthesis by a negative feedback control mechanism of possible importance in circumstances where intestinal bacterial dehydroxylation is increased.[139] Some patients with gallstones have increased hepatic HMG-CoA reductase activity associated with increased hepatic cholesterol concentration and possibly increased cholesterol secretion into the bile.[140] Chenodeoxycholate administration reverses this abnormality, lowering the HMG-CoA reductase level, resulting in a decrease of hepatic cholesterol synthesis.[140] Thus, the quality, in addition to quantity, of bile acids passing through the hepatocyte may have considerable influence on bile acid and cholesterol synthesis.[141] Conflicting evidence has been given regarding the effect of drug induction of 7α-hydroxylase activity.[142] [143]

Intestinal Transport and Absorption

The major bile acid conjugates form mixed micelles with polar and nonpolar Lipids, dependent on bile acid molar concentrations of 2mM to 3mM and a critical micellar temperature. This "dispersive" action explains their ability to transport cholesterol and amphipathic drug metabolites out of the body, and the acceleration of intestinal absorption of lipids and fat-soluble vitamins.[144] Bile acids participate in triglyceride hydrolysis by stabilizing emulsions and allowing maximum pancreatic lipase activity. Their implication as cofactors in extracellular cholesterol

esterase activity and protectors of the enzyme against proteolysis has been postulated.[145]

The human small intestine is relatively free of bacteria, and most bile acids are reabsorbed unchanged during the enterohepatic cycle. Maximal absorption (about 85%) of conjugated and unconjugated bile acids takes place in the terminal 60cm of ileum by means of a specific sodium-dependent active transport system.[141 146] A small amount of passive absorption of unconjugated and possibly glycine-conjugated bile salts occurs throughout the intestine. Obligatory anaerobes such as bacteroides and bifidobacteria, inhabiting the terminal ileum and colon, are responsible for deconjugation and degradation of a small portion, with the formation of secondary bile acids.[147] Bacterial degradation reactions include hydrolysis, 7α-dehydroxylation, and 7α-dehydrogenation. The production of secondary bile acids in the colon, leads to absorption, with reconjugation and/or sulfation in the liver.[148 149]

C. Estrogens

Physico-chemistry and Biological Functions

The naturally occurring estrogens contain 18 carbon atoms in the characteristic four ring cyclopentenophenanthrene structure with a phenolic C3 hydroxyl group.[150] Estrone, estradiol-17β and estriol were first isolated from

human pregnancy urine approximately forty years ago, Figure 4. Estrone and estriol are mainly products of estradiol-17β secreted by the ovarian follicle.[151]

Synthetic estrogens commonly incorporated into oral contraceptives include ethinyl-estradiol and its 3-methyl ester, 3-methyl-ethinyl-estradiol (mestranol), Figure 4. Progestogens used in these preparations are derivatives of either 19-nortestosterone or 17α-hydroxy-progesterone.[152]

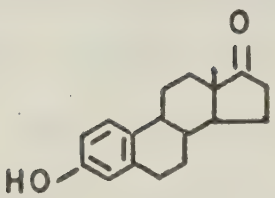
Estrogens are responsible for initiating and maintaining maturity of the female sex organs, secondary sex characteristics, the menstrual cycle and pregnancy. Their effects are widespread, encompassing lipid, carbohydrate, protein, nucleotide and electrolyte metabolism.[151 153] Liver microsomal "mixed function" oxidative enzymes are also affected by estrogens, which possibly act as competitive inhibitors.[100]

The estrogens have an affinity for specific hormone-receptor proteins in the cellular cytoplasm; the activated "hormone-receptor complexes" then concentrate in the cell nucleus, activating DNA (deoxyribonucleic acid), and initiating protein synthesis.[154]

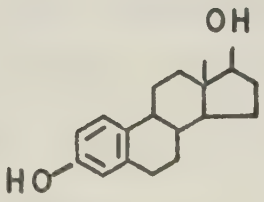
Biosynthesis

The major sites of estrogen production are the ovary and placenta with a minor contribution from the adrenal

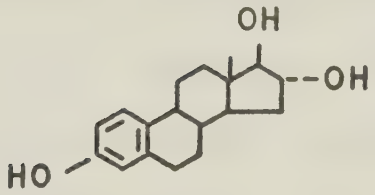
NATURAL ESTROGENS



ESTRONE

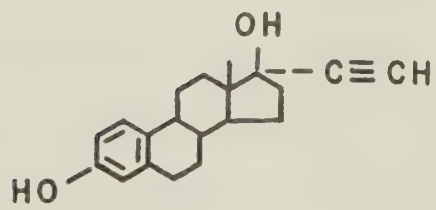


ESTRADIOL-17β

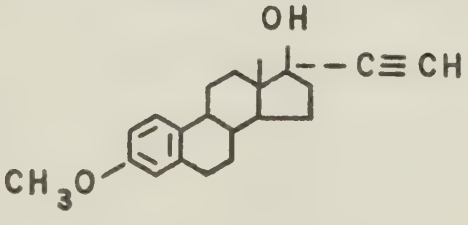


ESTRIOL

SYNTHETIC ESTROGENS



ETHINYL-ESTRADIOL



MESTRANOL

Figure 4. Natural and Synthetic Estrogens.

cortex. Biosynthesis is under pituitary control,[155] the main biosynthetic pathways formulated from numerous in vivo experiments are illustrated in Figure 5. Intracellular synthesis occurs in mitochondria and endoplasmic reticulum of cellular microsomes where a small rapidly metabolized cholesterol pool is associated with these organelles. Plasma free cholesterol is the preferred source to replenish this pool.[151]

The initial mitochondrial "desmolase" reaction is rate-limiting and consists of several steps involving hydroxylations requiring NADPH, which result in cleavage of the cholesterol side chain to form the C21 compound, Δ^5 pregnenolone. Subsequent hydroxylations at various positions of the sterol nucleus require NADPH, molecular oxygen and presumably cytochrome P450.

The mechanism of estrone and estradiol production from progesterone and pregnenolone involves dehydroepiandrosterone and testosterone. Estrone and estradiol are interconvertible; the enzyme, 17β-hydroxysteroid oxidoreductase, which catalyzes this conversion is found in many tissues, but 16α-hydroxylation of these estrogens to form estriol occurs mainly in the liver.[156]

Hepatic Metabolism

Some of the important pathways of estrogen metabolism in the liver are outlined in Figure 6, hydroxylation

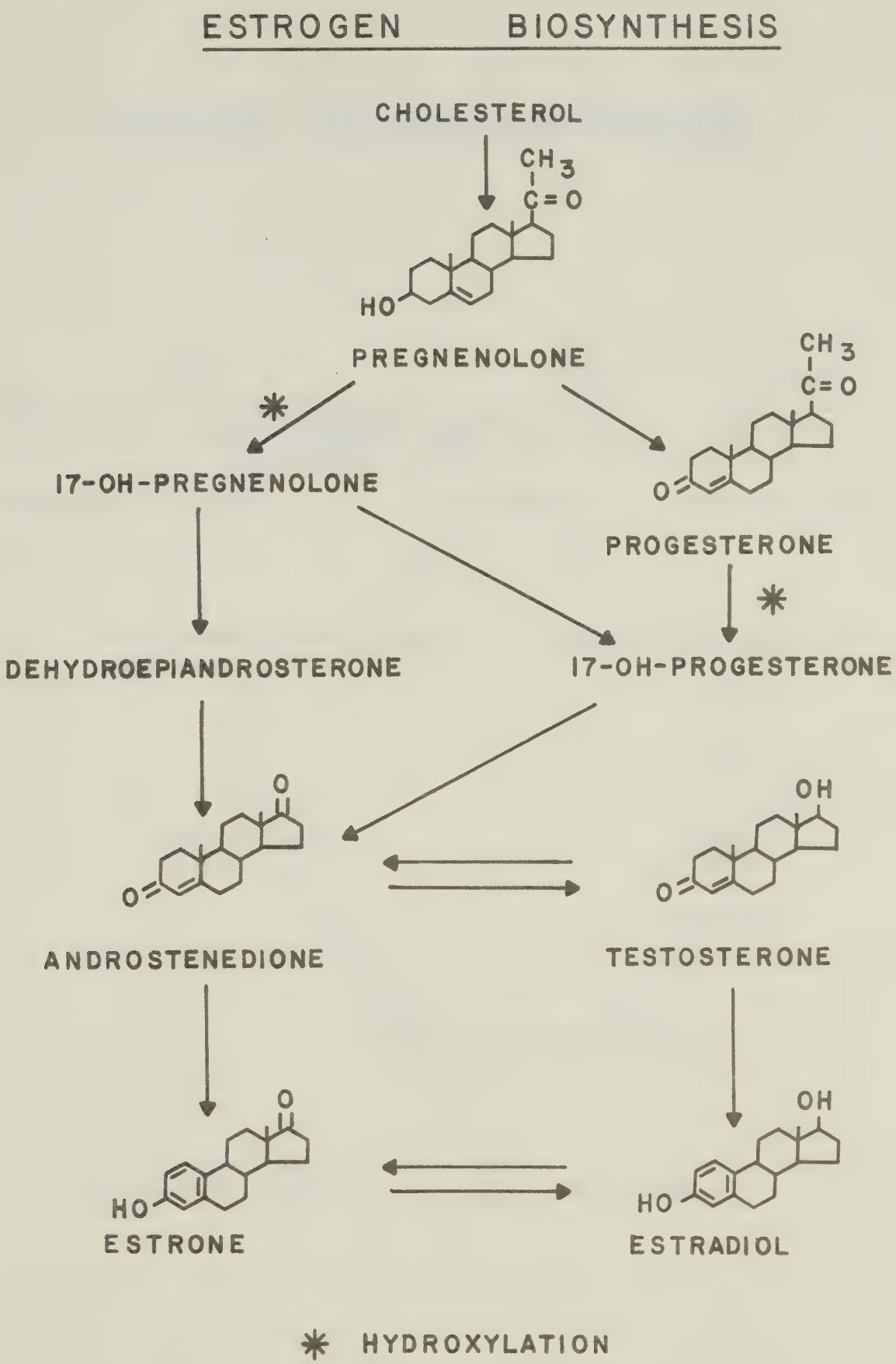


Figure 5. Estrogen Biosynthesis.

HEPATIC ESTROGEN METABOLISM

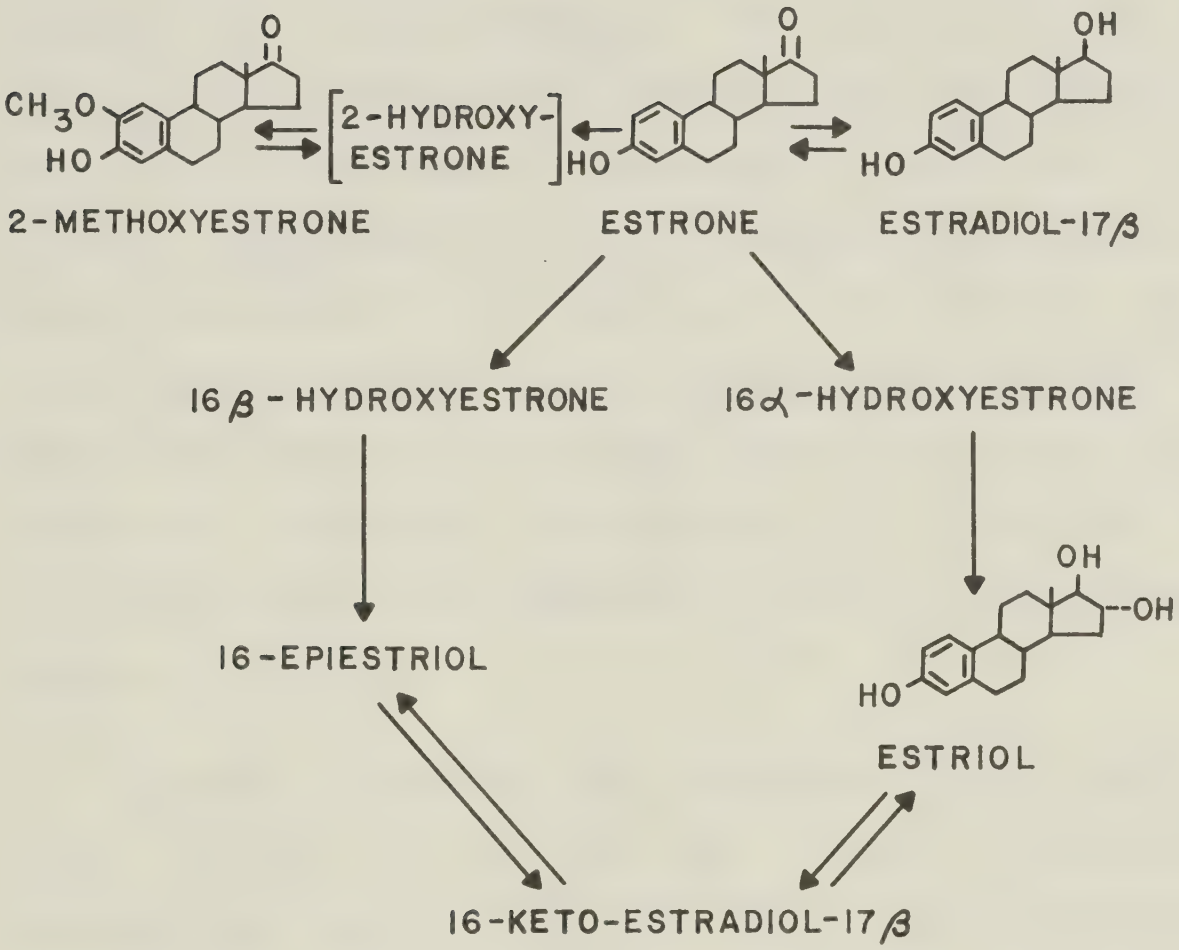


Figure 6. Hepatic Estrogen Metabolism.

occurring mainly at carbons 2, 6 and 16. Dehydrogenation of estradiol to estrone is faster than the reduction of estrone to estradiol, the equilibrium favoring estrone production. The main pathway for estrone metabolism is by way of 16α hydroxylation to 16α-hydroxyestrone, and subsequently to estriol. A second pathway involves C2 hydroxylation and the formation of 2-hydroxyestrone, which is then readily converted to 2-methoxyestrone. The estrogens and their metabolites are conjugated to glucuronides and sulfates in the 16 and 17 positions.[157] Glucuronides constitute the main estrogen conjugates,[158] but an appreciable proportion of estrone is excreted as the sulfate.[157 159] One half of the circulating estrogens may be secreted into the bile, with 80% of this fraction reabsorbed after hydrolysis in the intestinal lumen.[160] Reabsorption is associated with glucuronic acid reconjugation,[156] and a possible minor degree of sulfation.

Synthetic estrogens are metabolized in the liver in a manner similar to that of the natural estrogens.[157] Mestranol is first metabolized to ethinyl-estradiol, then hydroxylated at the C2 and C6 positions. The ethinyl group is unchanged. Progestogens are also metabolized in the liver by 10β-hydroxylation and C3 keto-reduction, with a small percentage converted into estrogens.[12] These synthetic hormones are then conjugated as glucuronates and sulfates, with subsequent secretion into the enterohepatic circulation and urine.[156]

Estrogens in Pregnancy

The placenta takes over from the ovary as the major source of estrogens derived mainly from fetal precursors, by the second trimester of pregnancy; a minor contribution comes from the maternal adrenal cortex. The fetal adrenal converts progesterone and pregnenolone to dehydroepiandrosterone, which is then hydroxylated and sulfated in the fetal liver to 16α-hydroxy-dehydroepiandrosterone sulfate. Subsequent conversion of the latter to estriol in the placenta, is the main pathway of estriol synthesis during pregnancy, Figure 7.

The placenta does not possess active 16α-hydroxylase and little 17α-hydroxylase, which consequently only allows estrogen synthesis from the C19 analogs, dehydroepiandrosterone, 16α-hydroxydehydroepiandrosterone, testosterone and androstenedione. These precursors reach the placenta as sulfated products, where active sulfatases release the free derivatives for conversion to estrogens.[161]

Other minor routes of estriol synthesis from steroids, occur in the maternal and fetal livers.[152] Factors controlling estrogen production during pregnancy are poorly understood,[162] but the increasing amounts of estrogens during pregnancy eventually overcome progesterone inhibition of the uterus resulting in the onset of labor. Estriol is the predominant urinary estrogen rising during pregnancy,

LEGEND FOR FIGURE 7

MA	Maternal adrenal cortex
ML	Maternal liver
MC	Maternal circulation
FA	Fetal adrenal cortex
FL	Fetal liver
FC	Fetal circulation
EHC	Enterohepatic circulation
CHOL	Cholesterol
AC	Acetate
PROG	Progesterone
PREG	Pregnenolone
DHA	Dehydroepiandrosterone
DHAS	Dehydroepiandrosterone sulfate
160HDHA	16 α -hydroxydehydroepiandrosterone
160HDHAS	16 α -hydroxydehydroepiandrosterone sulfate
E1	Estrone
E2	Estradiol
E3	Estriol
E1C	Estrone conjugate
E2C	Estradiol conjugate
E3C	Estriol conjugate
E3S	Estriol sulfate

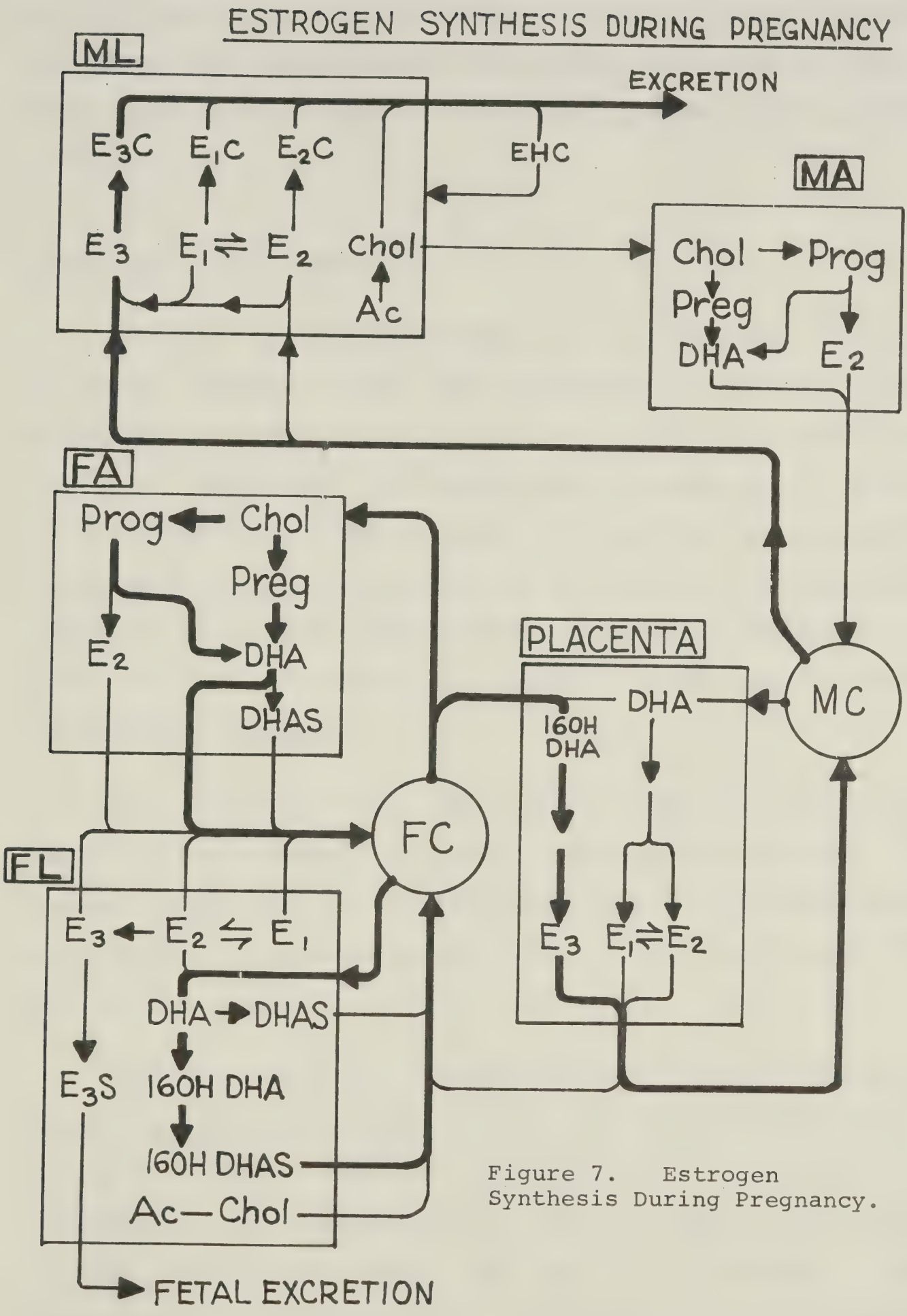


Figure 7. Estrogen Synthesis During Pregnancy.

falling quickly after parturition. Estrogen levels reflect placental and fetal function and intrauterine death has been associated with a fall in maternal urinary and plasma estrogens.[163]

Estrogens and the Liver

Extensive reviews of estrogen and progestogen effects on liver function have been presented, and it has been postulated that cholestasis induced by oral contraceptives may be attributed to constituent estrogens or their metabolites.[66 72 88] The standard BSP test is occasionally abnormal during oral contraceptive medication,[88 164] and the presence of a C17 alkylradical, a C3-oxo group, and a phenolic A ring, appear to be factors which induce this cholestatic effect.[12]

Oral contraceptives and synthetic estrogens have been noted for their effects on protein synthesis and changes in plasma lipids.[100 165 166] The latter may be associated with alterations in lipoprotein synthesis in the liver cell.[167 168 169]

Hepatic microsomal mixed-function oxidases catalyze oxidation and detoxication of many drugs and steroids. These reactions which include hydroxylation, dealkylation, oxidation, and sulfoxidation, require NADPH, molecular oxygen, and are dependent on P450.[100] Estradiol and progesterone are competitive inhibitors of these enzymes.

Norethynodrel enhances, whereas a combination of mestranol with norethynodrel reduces hexobarbital oxidation.[170] Large doses of certain drugs affect estrogen metabolism, and contraceptive steroids may have a dual effect on hepatic drug detoxication, inducing increased activities of certain enzymes and acting as competitive substrates.[100]

Ultrastructural studies of liver biopsies from women on oral contraceptives have revealed alterations of bile canaliculi, endoplasmic reticulum and mitochondria in the presence of clinically normal liver function.[171] Confirmation of these changes may be demonstrated in the increased ornithine carbamyl transferase (OCT) and induction of mitochondrial δ-aminolevulinic acid (ALA) synthetase. Estrogens can cause a massive increase in rough endoplasmic reticulum with accumulation of electron-dense granules in the Golgi apparatus in hepatocytes from female toads,[172] a change found in the liver of some patients who developed jaundice while taking oral contraceptives.[12]

Estradiol has no effect on bile flow in man and rat, whereas synthetic estrogens and estrone cause marked reductions.[62] A 50% reduction of bile output in the rat may result from increased permeability of the bile ductules and reduced choleresis.[56] Estrogens are also thought to have a direct inhibitory effect on hepatocyte bile salt excretion, interfering with secretion of conjugated cholates which are essential for micelle formation.[27] The conversion of cholate

to taurocholate in the rat is reduced by estrogens, which may contribute to cholestasis.[173]

There is no evidence that progesterone and its derivatives influence liver function and biliary excretion. However, some 19-nortestosterone derivatives may be inhibitory to this excretion either because they are C17 alkyl substituted, or because they are partially metabolized to estrogens.[12] Methyl testosterone can also have a damaging effect on the biliary canaliculus.[70]

CHAPTER III

MATERIALS AND METHODS

A. Patient and Control Groups

Normal Pregnant Women: Groups I, II, III, IV

A series of thirty-one pregnant women with a mean age of 25 years, ranging from 20 years to 32 years, were studied. Each woman was sampled on at least three occasions during pregnancy and once several days after parturition. Samples were grouped according to the gestational stage:

Group I - first trimester; up to 14 weeks gestation (average of 9 weeks).

Group II - second trimester; 14 to 28 weeks gestation (average of 21 weeks)

Group III - third trimester; 28 weeks to 40 weeks gestation (average of 33 weeks)

Group IV - post partum; 2 to 5 days (average of 3 days)

Women with Cholestasis of Pregnancy: Group V

This series of patients was collected over a two year period, and consisted of a total of twelve patients, some of whom were sampled several times. Patients were selected on a clinical basis and all were negative for Hepatitis B antigen. Three had a history of allergies, and one a history of jaundice during a previous pregnancy. The "cholestatic"

group included ten women in their third trimester of pregnancy, with a mean age of 24 years, ranging from 19 years to 31 years, four patients with pruritus gravidarum, and six patients with pruritus and cholestasis. Two of these six women had cholelithiasis which led to cholecystectomy several weeks after parturition. A liver biopsy from one of these patients revealed evidence of canalicular dilation and the presence of inspissated bile plugs, but no significant inflammatory changes.

Two women who developed pruritus during the second trimester were studied, but excluded from Group V. We were able to follow one of the six cholestatic patients throughout her second pregnancy. She first developed jaundice while taking Ortho-180 (1mg norethindrone, 0.08mg mestranol), with subsequent recurrence during her first and second pregnancies.

Women Using Oral Contraceptives: Group OC

This group consisted of seventeen healthy women of child-bearing age, with a mean age of 24 years, ranging from 18 years to 43 years, all of whom were taking oral contraceptives containing a progestogen in combination with either ethinyl-estradiol or mestranol.

Normal Non-pregnant Controls: Group C

This was a group of thirty-three normal healthy women

with a mean age of 29 years, ranging in age from 20 years to 45 years.

Jaundiced Non-pregnant Patients

A series of twenty-six hospitalized patients with jaundice, were randomly selected for serum total bile acid estimations. The purpose of this was to make comparisons with Group V patients and to check sensitivity of the method.

B. Samples

Blood samples were obtained whenever possible from fasting individuals. This proved difficult, however, with normal pregnant women when they had afternoon appointments with the obstetrician. Whole blood (30ml) was drawn by venipuncture into B-D vacutainer tubes, allowed to clot, then centrifuged at low speed. One portion of the serum was analyzed within 3 h to 4 h for liver enzymes, total bilirubin, lipids and lipoproteins. A second portion of serum was frozen and stored at -20° for subsequent analyses of total bile acids and estrogens.

C. Liver Enzymes and Bilirubin

Routine liver function tests were performed on serum samples by sequential multichannel analysis (SMA 12/60), according to accepted Technicon methodology.[174] Additional

estimations of serum 5'-nucleotidase[175] and alanine aminotransferase[176] were carried out according to modified procedures.

D. Serum Lipids and Lipoproteins

Triglycerides[177] and cholesterol[178] were determined simultaneously from an isopropanol extract by means of a semi-automated Technicon procedure. Serum lipoprotein fractions were separated by electrophoresis in agarose gel at pH 8.6, then visualized after staining with Sudan Black B, according to the procedure previously reported from this laboratory.[179]

E. Lipoprotein-X (LP-X)

On electrophoresis in agarose gel, LP-X migrates towards the anode with the β-lipoproteins. In agar gel, however, this abnormal lipoprotein migrates towards the cathode, thus facilitating its separation from other serum lipoproteins.[35]

Electrophoresis of serum samples was carried out in 1% "bacto-agar" gel* using barbital buffer (pH 8.6, ionic strength 0.05), according to Seidel.[180] A faster, simpler method for visualization of LP-X was tried,[181] utilizing the principle of cathodal polyanionic precipitation of LP-X.

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* Difco Labs, Detroit, Mich.

F. Total Bile Acids

Serum total bile acids were quantitated by an enzymatic-fluorimetric technique[182] after liquid-solid extraction with non-ionic amberlite XAD-2 resin,[94] and the removal of interfering lipids by high-speed centrifugation.

The amount of $NADH.H^+$ generated from NAD^+ and 3α-hydroxy-bile acids in the presence of 3α-hydroxysteroid dehydrogenase,* was measured fluorimetrically at 460nm in an Aminco-Bowman spectrophotofluorometer after activation at 350nm. This reaction may be represented by the following equation.

$$3\alpha\text{-Hydroxysteroid} + NAD^+ \underset{pH^5}{\overset{pH^9}{\rightleftharpoons}} 3\text{-Ketosteroid} + NADH + H^+$$

An alkaline pH and the use of hydrazine sulfate as a ketone trapping agent, shifted the reaction to the right, making it complete. A standard graph was prepared with each assay and the amount of fluorescence related back to the original quantity of bile acid present in a sample.

Minor modifications were made to the method. Diluted samples were sonicated for one minute prior to extraction,

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* Worthington - highly purified 3α-hydroxysteroid dehydrogenase. (E.C. 1.1.1.50.) Code: STDHP. Prepared from cells of Pseudomonas Testeroni. Minimum activity:0.5IU per ng. 1 unit of activity is the amount of enzyme, which will reduce 1umol of NAD^+ per minute at 25° and pH 9.5.[183]

alleviating protein adsorption and subsequent loss of bile acids. Column flow rates were controlled at 0.2 - 0.4 ml/min by means of a proportioning pump, and 95% ethanol (redistilled at 78°) was used to elute bile acids from the XAD-2 resin. Two high-speed centrifugations were introduced, to eliminate interfering neutral lipids; the first was at 34,800 x g for 20 min before the enzymatic assay, and the second at 27,000 x g for 10 min prior to the fluorimetric measurement. A range of standards containing 0.00125umol to 0.020umol of glycocholate were prepared by addition to portions of a pooled serum XAD-2 extract, in order to compensate for fluorescence quenching.

Percentage recoveries of glycocholate from a serum control and reagent blank were determined with each assay to check accuracy and losses during XAD-2 extraction, and these showed mean ± SD* values of 81% ± 17% when n* was 43. Percentage recoveries of chenodeoxycholate, determined during initial method development were 82% ± 12% (Mean ± S.D.), n=51; and 88% ± 9% when ^{3}H-cholic acid was added to 15 serum samples.

The standard curve showed daily reproducibility and the sensitivity and precision of the method are outlined as follows:

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* SD = standard deviation
  n = number of values
* C.V. = coefficient of variation
  $\bar{X}$ = mean

-Sensitivity was 0.001 um/cuvette, or 2.0umol/1 when 2.0ml serum were extracted.

-Within assay precision, calculated from 14 pairs of duplicates, showed a:

SD of 0.9umol/1
CV* of 16.3%
$\bar{X}$* of 6.0umol/1

-Between assay precision, based on 13 pairs of duplicates of control and patient sera, showed a:

SD of 1.8umol/1
CV of 23.3%
$\bar{X}$ of 7.7umol/1

G. Unconjugated Estrogens

Unconjugated estrone(E1), estradiol(E2) and estriol(E3), were each measured in serum by similar independent radioimmunoassay procedures. Methodology was performed according to P. Krahn (personal communication), who modified existing procedures for quantitating these steroids.[184] [185] Highly specific rabbit antisera to each estrogen, prepared from estrogen - 6 - carbioxime - bovine serum albumin conjugates, were utilized.*

In theory, a sample extract, containing an unknown quantity of unlabelled antigen is incubated with a specific antibody in the presence of a small known amount of labelled antigen. Both labelled and unlabelled antigens compete for a

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* Purchased from K & T Biological Services, Edmonton, Alta.

limited number of binding sites on the antibody.

The presence of a high concentration of antigen (estrogen) in the sample, means there is less binding of labelled antigen.

Reagents used for each estrogen assay were:

1. Phosphate Buffered Saline-Bovine Serum Albumin (PBS-BSA) pH 7.4: contains 0.01 M K_2HPO_4, 0.15 M NaCl, 0.02M NaN_3, 0.1g/dl BSA in deionized water.
2. Stock Standards: 10mg/ml
   Estrone, estradiol or estriol prepared in deionized water.
3. Working Standards of 0 to 600pg:
   Prepared by diluting stock standard with PBS-BSA.
4. ^{3}H-Estrone: (6,7-^{3}H(N)) - sp. act. 85-105 Ci/mmol
   ^{3}H-Estradiol: (2,4,6,7,^{3}H(N)) - sp. act. 85-105 Ci/mmol
   ^{3}H-Estriol: (2,4,6,7-^{3}H(N)) - sp. act. 85-105 Co/mmol
   The three labelled estrogens (New England Nuclear) were diluted to a specific activity of 0.5uCi/10.5ml PBS-BSA.
5. Antibody: (K & T Biological Services)
   reconstituted in 10.2ml PBS-BSA
6. Dextran-coated charcoal (DCC):
   2.5g Noritt A (Neutral) (Fisher Scientific) and 0.25g dextran (M.W 60,000-90,000) (Canlab), mixed with 100 ml PBS-BSA, and stored at 4°.
7. Methylene Chloride:
   Reagent grade, - redistilled once at 38°.

8. Scintillation Fluid: "Unogel". (Schwarz/Mann) Becton Dickinson Orangeburg, N.Y.

Extraction and Recovery

Estimations of patients' serum estrogen levels were made according to gestational maturity, and volumes varying from 0.05ml to 2.0ml were diluted to 2.0ml with PBS-BSA, then extracted. Three successive one minute extractions were performed, using 5.0ml methylene chloride each time, and a recovery was estimated by the addition of a trace amount of label (400cpm) to each sample. A reagent blank was prepared from 15ml methylene chloride, and a unogel "blank" counted for "background" correction. Extracts were taken to dryness at 45° under nitrogen, reconstituted in 1.0ml of PBS-BSA, and 0.2ml of each extract was transferred to a scintillation vial containing 10ml of "unogel" for a recovery determination. Radioimmunoassay was performed on the remaining portion of serum extracts. An "input recovery" vial was prepared, which contained 10ul ^{3}H-estrogen, and 0.2ml PBS-BSA in 10ml "unogel".

Radioimmunoassay (RIA)

RIA determinations were performed in duplicate in 10 x 75mm disposable culture tubes.* See Table 1. All tubes were

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* Canlab

Table 1. Estrogen Radioimmunoassay Procedure

	Total Counts Reference ml	Non-Specific Binding ml	Reagent Blank ml	Standard ml	Sample ml
PBS-BSA	1.30	0.70	0.50	0.60	0.50
Standards (0-600pg)	-	-	-	0.10	-
Reagent Extract	-	-	0.20	-	-
Sample Extract	-	-	-	-	0.20
^{3}H-estrogen	0.10	0.10	0.10	0.10	0.10
Estrogen Antibody	-	0.10	0.10	0.10	0.10
DCC	-	0.50	0.50	0.50	0.50

mixed well, incubated at room temperature for 1h, then chilled to 4° in ice water for 10 min. Tubes containing "total reference counts" were decanted into scintillation vials containing 10ml of unogel. DCC was added to all remaining tubes and 5 min allowed for adsorption. Centrifugation for 10 min at 3000rpm provided a clear supernatant which was decanted into a scintillation vial containing 10ml unogel. All vials were mixed and counted for 10 min (2% efficiency) in a Searle Isocap 300/Liquid Scintillation counter.

Calculations

The standard curve of % bound vs pg estrogen was plotted on linear graph paper. Calculations were:

$$\% \text{ Recovery} = \frac{(\text{Recovery-Background}) \text{ cpm} \times 1.0 \times 100}{(\text{Recovery Input-Background}) \text{ cpm} \times 0.2}$$

$$\text{ng/ml Estrogen} = \frac{(\text{pg}) \text{ estrogen} \times 1.0 \times 100}{\% \text{ Recovery} \times 0.2 \times \text{ml serum} \times 1000}$$

Sensitivity, Accuracy, Precision, Specificity

Sensitivity of each estrogen method was 50pg. The working range for the standard curve was from 50 to 600pg for estradiol and estriol, and 100 to 600pg for estrone. Standard curves were reproducible.

Percent recoveries showed mean ± SD values of:

72% ± 14% for estrone when n was 207

80% ± 15% for estradiol when n was 203

67% ± 12% for estriol when n was 207

Within assay precision calculated from 20 samples run in duplicate on the same day was:

	Estrone	Estradiol	Estriol
SD	0.8ng/ml	0.9ng/ml	0.2ng/ml
CV	6.7%	7.7%	3.0%
$\bar{X}$	11.9ng/ml	12.7ng/ml	8.4ng/ml

Between assay precision based on duplicates from control and patient sera carried through the entire procedure and assayed on different days, was:

	Estrone	Estradiol	Estriol
SD	0.5ng/ml	1.1ng/ml	0.3ng/ml
CV	17.8%	16.6%	16.6%
$\bar{X}$	2.8ng/ml	6.6ng/ml	1.8ng/ml
n	18	22	26

Specificities of antisera to estradiol and estriol were high. Estradiol, however, cross-reacted with estrone antiserum, requiring corrections to the estrone measurements. See Table 2.

Table 2. Antisera Specificity:

% Cross-Reaction at 25% Binding

Steroid	Antiserum To Estrone	Estradiol	Estriol
Estrone	100	0.8	<0.01
Estradiol-17β	25	100	0.5
Estriol	0.2	0.8	100
Estrone-3-Sulfate	0.2	<0.1	0.3
Progesterone Cortisol Dehydroepiandrosterone Testosterone	<0.1	<0.1	<0.001

H. Statistical Analysis

The mean (X), standard deviation (SD), coefficient of variation (CV), and standard error of the mean (SEM) were calculated for each group of data, on an Olivetti programma 101 calculator, using the following formulae.

$$\bar{X} = \frac{\Sigma X}{n} \; ; \quad SD = \sqrt{\frac{\Sigma (X-\bar{X})^2}{n-1}} = \sqrt{\frac{\Sigma (X^2) - \Sigma X^2/n}{n-1}}$$

$$CV = \frac{SD \times 100}{\bar{X}} \; ; \quad SEM = \frac{SD}{\sqrt{n}}$$

n = number of values
Σ = sum of

Within and between assay precision of duplicates were determined by:

$$SD = \sqrt{\frac{d^2}{n}} \qquad d^2 = (\text{difference between duplicates})^2.$$

The student 't' test for unpaired data was used to determine the significance of differences between mean values. Differences were considered to be significant at a probability (P) value of 0.05 (5%) or less, and highly significant when P was less than 0.001 (0.1%). A trend towards a difference was indicated by P values of 0.10 or less.

$$t = \frac{\text{difference between 2 means}}{\text{standard error of mean difference}} = \frac{\bar{X}d}{Sd}$$

$$Sd = \sqrt{\frac{SD_1^2}{n1} + \frac{SD_2^2}{n2}}$$

Probabilities for calculated 't' values were obtained for the appropriate "degrees of freedom" (df), from a "t" table (Chemical Rubber Co. Handbook of Statistics).

df = (n1 + n2) - 2 = the number of independent random selections which may be made from a "population".

CHAPTER IV

RESULTS

A. Liver Function Tests, Lipids, Lipoproteins

Mean values, standard deviations and ranges of values for serum liver function tests and lipid analyses of the groups studied are shown in Tables 3,4,5,6 Normal ranges of values established in this laboratory are:

- alkaline phosphatase: 20-90 IU/l.
- serum aspartate aminotransferase (SGOT): 10-50 IU/l.
- serum alanine aminotransferase (SGPT): 5-50 IU/l.
- lactate dehydrogenase (LDH): 90-200 IU/l.
- 5'-nucleotidase: 2-14 IU/l.
- Total bilirubin: 0.2-1.5 mg/dl.
- Triglycerides: 50-150 mg/dl.
- Cholesterol: 75-250 mg/dl.

Results for nonpregnant controls and women using oral contraceptives were within normal limits. One woman using Ovaral (0.25 mg norgestrel 0.05 mg ethinyl estradiol), had slightly raised SGOT and SGPT levels.

Serum alkaline phosphatase and triglycerides showed the most striking changes during normal pregnancy, and these were larger in pregnancy with associated cholestasis when other liver function tests showed abnormalities. A lipoprotein band with electro-phoretic mobility between the β and pre-β regions was demonstrated in 13 normal

LEGEND FOR TABLES 3,4,5,6,13

ALP	Alkaline phosphatase
T.BILI	Total bilirubin
LDH	Lactate dehydrogenase
SGOT	Serum aspartate aminotransferase
SGPT	Serum alanine aminotransferase
5'-NT	5'-nucleotidase
TRIG	Triglycerides
CHOL	Cholesterol
LIPOS	Lipoproteins
LP-X	Lipoprotein-X
1 IU/l	That amount of enzyme which will convert 1 umol of substrate into products per minute under standard conditions.

Table 3. Liver Function Tests, Lipids and Lipoproteins for Nonpregnant Controls (Group C) and Women Using Oral Contraceptives (Group OC).

Test	Group C (n=33)			Group OC (n=17)		
	Mean	SD	Range	Mean	SD	Range
ALP (IU/l)	56	15	36-95	50	14	33-79
T.BILI (mg/dl)	0.5	0.2	0.2-1.0	0.4	0.2	0.2-0.7
LDH (IU/l)	154	18	120-198	162	23	90-200
SGOT (IU/l)	22	4	15-35	29	11	10-50
SGPT (IU/l)	11	5	5-26	17	18	7-79
5'NT (IU/l)	6.3	2.1	2.6-10.7	8.6	2.8	3.8-14.0
TRIG (mg/dl)	75	29	27-162	96	33	61-182
CHOL (mg/dl)	201	36	138-291	182	33	143-259
LIPOS	All normal			All normal		
LP-X	All negative			All negative		

Table 4. Liver Function Tests, Lipids and Lipoproteins for Normal First Trimester (Group I) and Second Trimester (Group II) Pregnancy.

Test	Group I (n=25)			Group II (n=30)		
	Mean	SD	Range	Mean	SD	Range
ALP (IU/l)	56	14	37-74	72	24	38-128
T.BILI (mg/dl)	0.5	0.3	0.2-1.6	0.4	0.3	0.1-1.8
LDH (IU/l)	158	14	132-190	164	28	127-255
SGOT (IU/l)	22	8	12-47	24	16	15-32
SGPT (IU/l)	18	8	3-36	18	11	7-43
5'NT (IU/l)	7.2	3.7	3.0-16.2	11.0	10.0	3.0-24.9
TRIG (mg/dl)	101	34	52-166	178	44	92-233
CHOL (mg/dl)	163	24	109-217	215	39	159-309
LP-X	All negative			All negative		

Table 5. Liver Function Tests, Lipids and Lipoproteins for Normal Third Trimester Pregnancy (Group III) and the Early Pueperium (Group IV).

Test	Group III (n=28)			Group IV (n=16)		
	Mean	SD	Range	Mean	SD	Range
ALP (IU/l)	150	79	69-324	152	31	107-220
T.BILI (mg/dl)	0.4	0.2	0.1-1.1	0.4	0.1	0.2-0.6
LDH (IU/l)	185	34	114-266	233	61	132-300
SGOT (IU/l)	26	9	13-64	37	16	20-64
SGPT (IU/l)	17	9	7-55	19	7	12-38
5'NT (IU/l)	8.8	2.9	3.9-14.9	10.0	3.9	3.5-20.2
TRIG (mg/dl)	234	65	122-363	226	76	128-405
CHOL (mg/dl)	239	47	129-366	214	42	139-300
LIPOS	13 had intermediate Band			10 had intermediate Band		
LP-X	All negative			All negative		

Table 6. Liver Function Tests, Lipids and Lipoproteins for Third Trimester Patients with Pruritus Gravidarum and/or Cholestasis of Pregnancy (Group V).

Test	Group V (n=10)		
	Mean	SD	Range
ALP(IU/l)	303	68	134-583
T.BILI(mg/dl)	1.2	0.7	0.3-2.6
LDH(IU/l)	179	46	120-258
SGOT(IU/l)	109	111	20-339
SGPT(IU/l)	96	120	17-374
5'NT(IU/l)	23.1	11.0	11.1-42.7
TRIG(mg/dl)	365	110	218-550
CHOL(mg/dl)	289	55	233-358
LIPOS	10 had intermediate band		
LP-X	3 were +		

pregnancies during the third trimester. This was termed "intermediate band", and was found in sera from all ten patients in Group V, cholestasis of pregnancy. Lipoprotein-X was seen in the sera from three of the Group V patients, one of whom had clinical cholelithiasis.

B. Total Bile Acids

Results for total serum bile acids for the groups studied are shown in Table 7 and graphically presented in Figure 8. Total bile acids increased gradually during normal pregnancy falling slightly in the early pueperium. Differences between sample means of the first and third trimester were significant ($P<0.01$), whereas those between the first and second trimesters showed a trend towards significance ($P<0.10$). There was no significant difference between the second and third trimesters. Mean differences between controls and each of the pregnancy groups were highly significant ($P<0.001$). However, no difference could be found between means for "controls" and women taking oral contraceptives.

Total bile acid values in serum from women with cholestasis of pregnancy, were up to ten times those values found during normal pregnancy. Mean concentrations were similar for this group of women and a group of clinically "jaundiced control" patients, with a wide range in values

Table 7. Serum Total Bile Acids (umol/l)

Group		n	Mean	SD	Range
Non-Pregnant					
Controls - C		33	4.2	2.7	0.4 - 13.0
On Oral - OC Contraceptives		17	3.7	1.7	1.1 - 8.0
Jaundiced Controls		26	40.7	42.0	1.6 - 178
Normal Pregnant					
Trimester	I	26	8.3	3.9	1.5 - 18.2
Trimester	II	31	10.6	6.9	2.4 - 33.7
Trimester	III	31	11.9	6.4	3.0 - 33.2
Post-Partum	IV	15	9.9	6.4	2.5 - 12.6
Cholestatic Pregnant	V	10	39.6	41.9	1.6 - 125

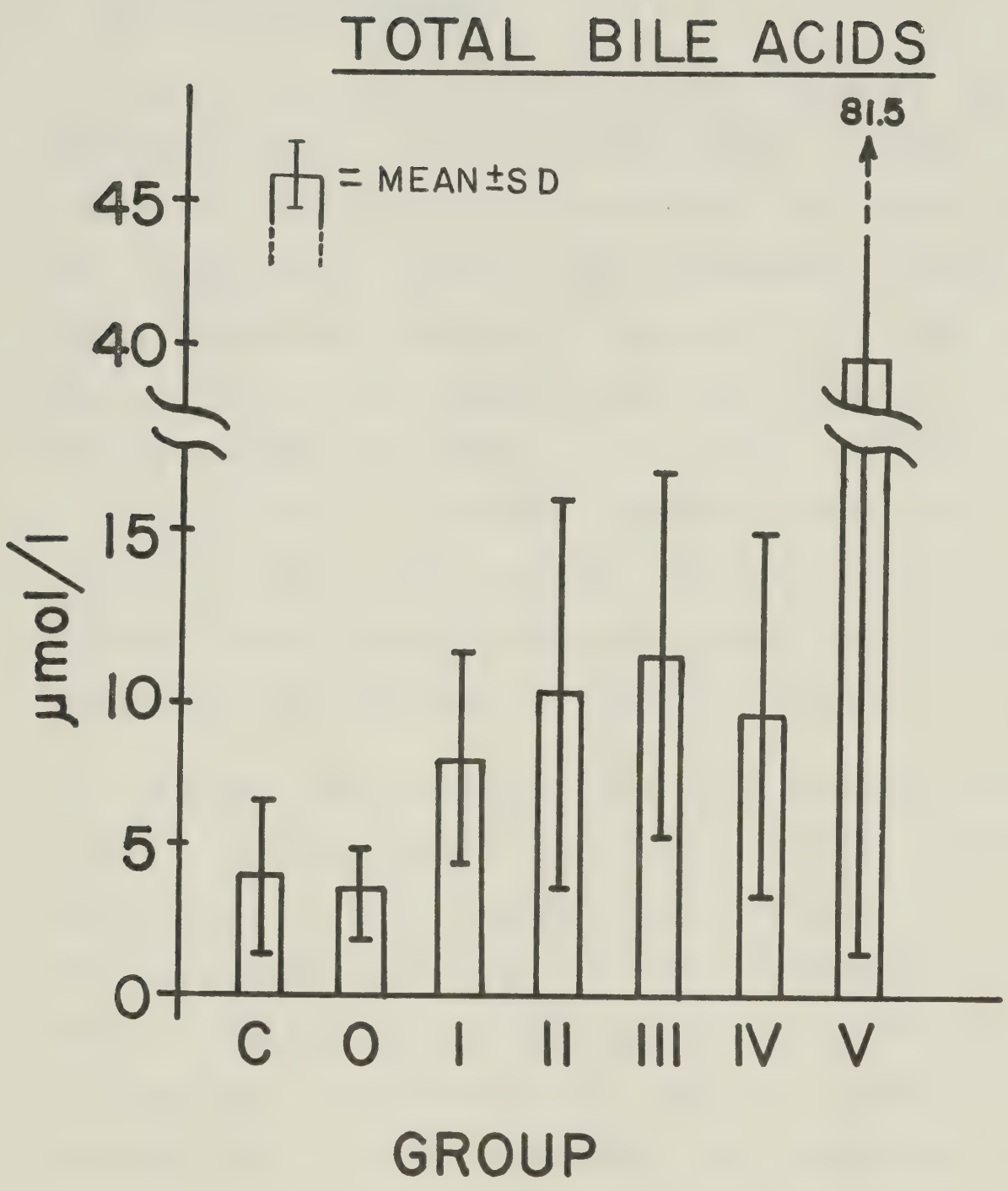

Figure 8. Total Serum Bile Acids.

for both groups. Values for subjects with pruritus gravidarum were similar to those of normal pregnant women.

C. Unconjugated Estrogens

Total, and each of the three component estrogens increased during pregnancy and dropped rapidly after parturition (Tables 8,9,10,11; Figures 9,10). Total estrogen mean differences between each trimester, (Table 8) were highly significant ($P<0.001$). Estradiol increased at the fastest rate, with values which were higher ($P<0.01$) than those of estrone and estriol by the second trimester (Tables 9,10,11). Estriol increased steadily from the first trimester until term, whereas estrone showed the lesser increase during the third trimester. Following parturition, estrone was the dominant unconjugated estrogen.

The mean total estrogen value shown for the cholestatic Group V subjects was significantly higher, and higher estrone values were present when compared with normal pregnant women from each of the trimesters ($P<0.005$). Estrone levels were higher than estradiol and estriol levels in pregnancy cholestasis and pruritus gravidarum, in contrast to normal pregnancy. The difference between estradiol and estriol mean values was not significant, and no significant difference could be found for estradiol and estriol between cholestasis of pregnancy and normal third trimester pregnancy.

Table 8. Serum Total Unconjugated Estrogens (ng/ml)

Group		n	Mean	SD	Range
Non-Pregnant					
Controls - C		33	0.6	0.3	0.1 - 1.3
On Oral - OC Contraceptives		17	0.3	0.3	0.1 - 1.6
Normal Pregnant					
Trimester	I	26	5.0	3.6	1.0 - 16.9
Trimester	II	26	26.7	12.4	4.6 - 56.2
Trimester	III	26	45.2	17.2	21.2 - 88.6
Post-Partum	IV	17	2.4	2.4	0.4 - 9.2
Cholestatic Pregnant	V	10	55.7	18.8	27.8 - 84.3

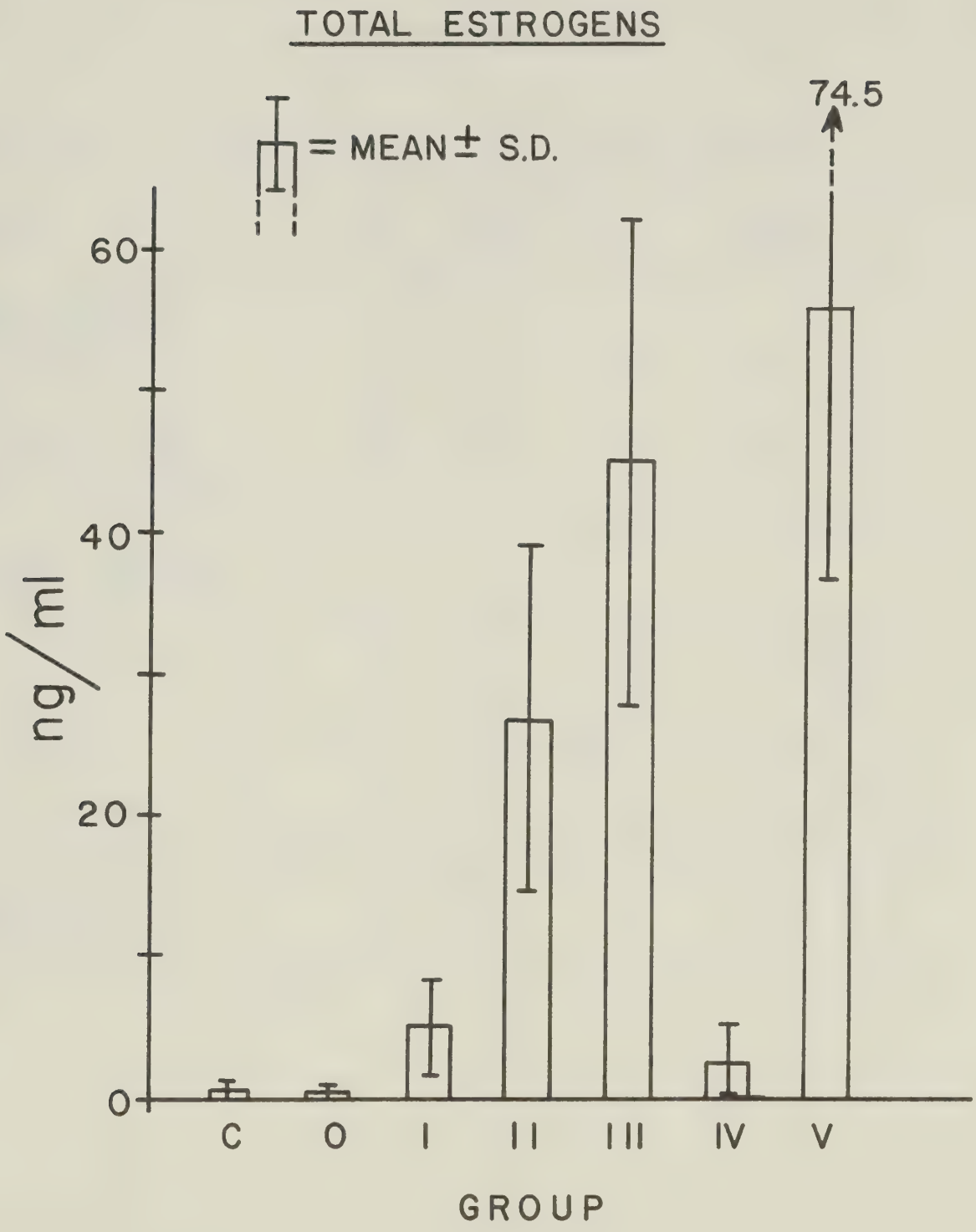

Figure 9. Total Serum Unconjugated Estrogens.

Table 9. Serum Unconjugated Estrone (ng/ml)

Group		n	Mean	SD	Range
Non-Pregnant					
Controls - C		33	0.4	0.3	0 - 1.3
On Oral - OC Contraceptives		17	0.2	0.3	0 - 1.3
Normal Pregnant					
Trimester	I	26	1.8	2.2	0 - 4.9
Trimester	II	26	7.5	7.6	0 - 30.9
Trimester	III	26	10.8	10.0	0 - 42.2
Post-Partum	IV	17	1.5	2.3	0 - 7.9
Cholestatic Pregnant	V	10	26.7	15.6	8.6 - 56.0

Table 10. Serum Uncongugated Estradiol (ng/ml)

Group		n	Mean	SD	Range
Non-Pregnant					
Controls - C		33	0.2	0.1	0 - 0.6
On Oral - OC Contraceptives		17	0.1	0.1	0 - 0.2
Normal Pregnant					
Trimester	I	26	2.2	2.2	0.4 - 10.8
Trimester	II	26	12.4	5.8	3.0 - 23.8
Trimester	III	26	20.7	10.7	0.1 - 53.9
Post-Partum	IV	17	0.2	0.1	0.1 - 0.4
Cholestatic Pregnant	V	10	16.9	6.4	6.5 - 31.3

Table 11. Serum Unconjugated Estriol (ng/ml)

Group		n	Mean	SD	Range
Non-Pregnant					
Controls - C		33	0.1	0.1	0 - 0.3
On Oral - OC Contraceptives		17	0.1	0.1	0 - 0.3
Normal Pregnant					
Trimester	I	26	0.8	0.4	0.1 - 1.6
Trimester	II	26	7.0	4.4	2.0 - 24.2
Trimester	III	26	13.1	6.2	4.3 - 22.3
Post-Partum	IV	17	0.7	0.3	0.2 - 1.4
Cholestatic Pregnant	V	10	12.1	5.0	7.3 - 21.0

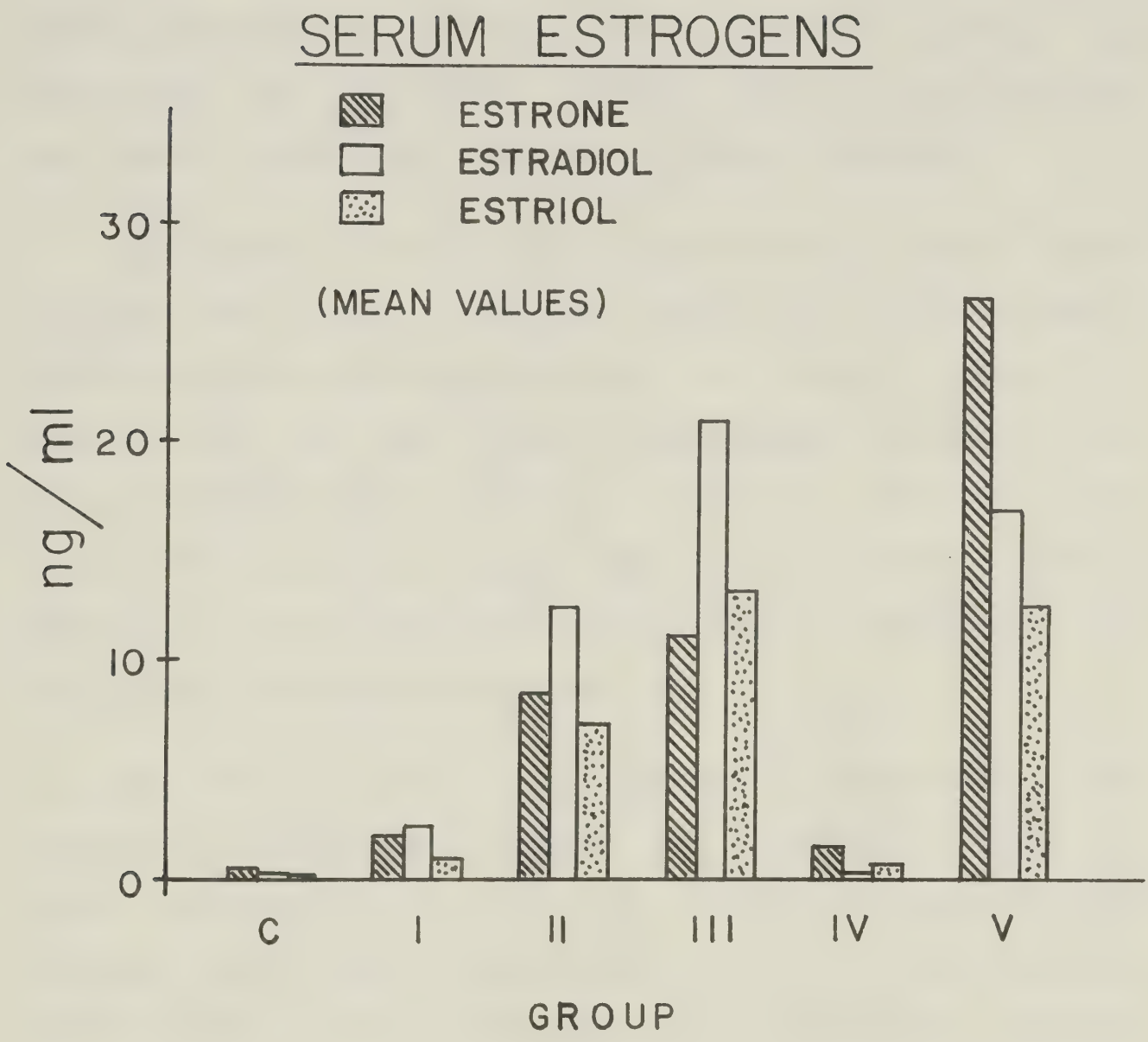

Figure 10. Individual Serum Unconjugated Estrogens.

When the mean relative percentages of total for each unconjugated estrogen were compared (Figure 11) no significant difference was found for estrone, estradiol and estriol between each of the normal pregnancy groups. In cholestasis of pregnancy, however, estrone and estradiol were out of proportion compared to normal pregnancy.

Nonpregnant controls had a significantly higher total estrogen mean value than women using oral contraceptives, despite very low concentrations for both groups (Table 8). Estrone was the main serum unconjugated estrogen, with similar concentrations for both groups. The estradiol mean value for controls was higher than that of estriol, whereas there was no mean difference between the two estrogens for the group on oral contraception.

In summary, mean total estrogen levels showed that estrone was significantly higher in cholestasis, than in normal pregnancy, and was the predominant unconjugated estrogen in the former group, whereas estradiol was dominant during normal pregnancy. The relative percentage of estrone was twice, whereas estradiol was one half that of normal third trimester pregnancy. The mean total estrogen level was lower in women on oral contraceptives than in normal controls due to a lowered mean estradiol value.

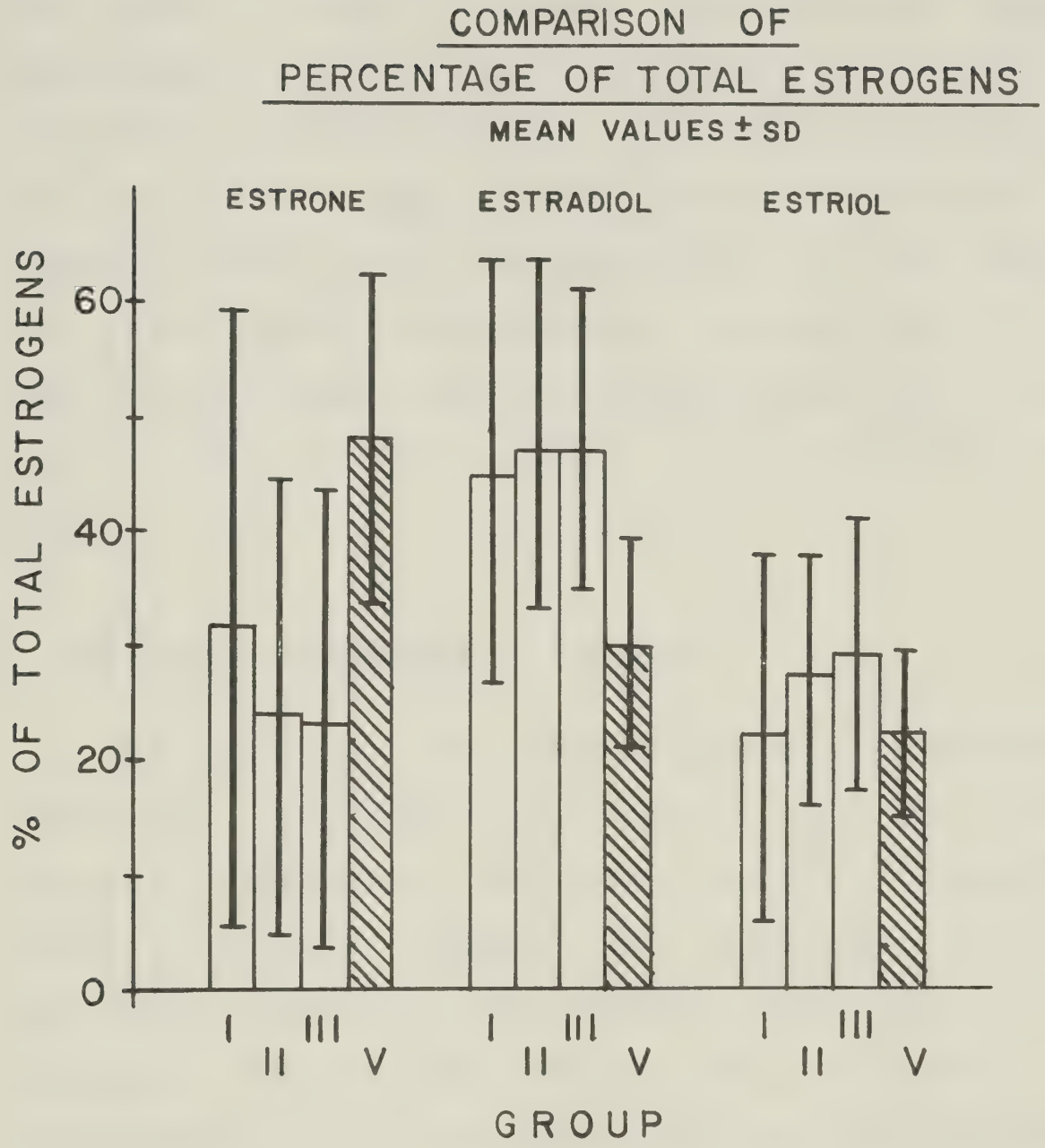

Figure 11. Comparison of Percentage of Total Estrogens.

D. Ratios of Total Bile Acids to Total Unconjugated Estrogens

Figure 12 shows a gradual drop in mean ratio of total bile acids to total estrogens during normal pregnancy, illustrating a faster rate of increase of estrogens than bile acids. The mean ratio for cholestasis of pregnancy was similar to that seen in normal pregnancy during the third trimester, even though concentrations for both variables were much higher in cholestasis. The mean ratio of 8 to 1, shown by the control group was significantly less than the ratio of 19 to 1 for women on oral contraceptives (Table 12).

E. Recurrent Cholestasis of Pregnancy

Comparison of the normal findings, illustrated in Figure 13, with those of patient C.B., who developed recurrent cholestasis, and was followed throughout her second pregnancy (Figure 14, Table 13) demonstrates significant differences. The abnormal findings at 13 weeks' gestation were a high estrone and a slightly raised triglyceride. By 25 weeks' gestation, estrone had dropped to the second trimester range of values, whereas estradiol showed slightly elevated levels. At 35 weeks' gestation, total serum bile acids were increased and 0.5 mg/dl of this patient's total serum bilirubin was conjugated.

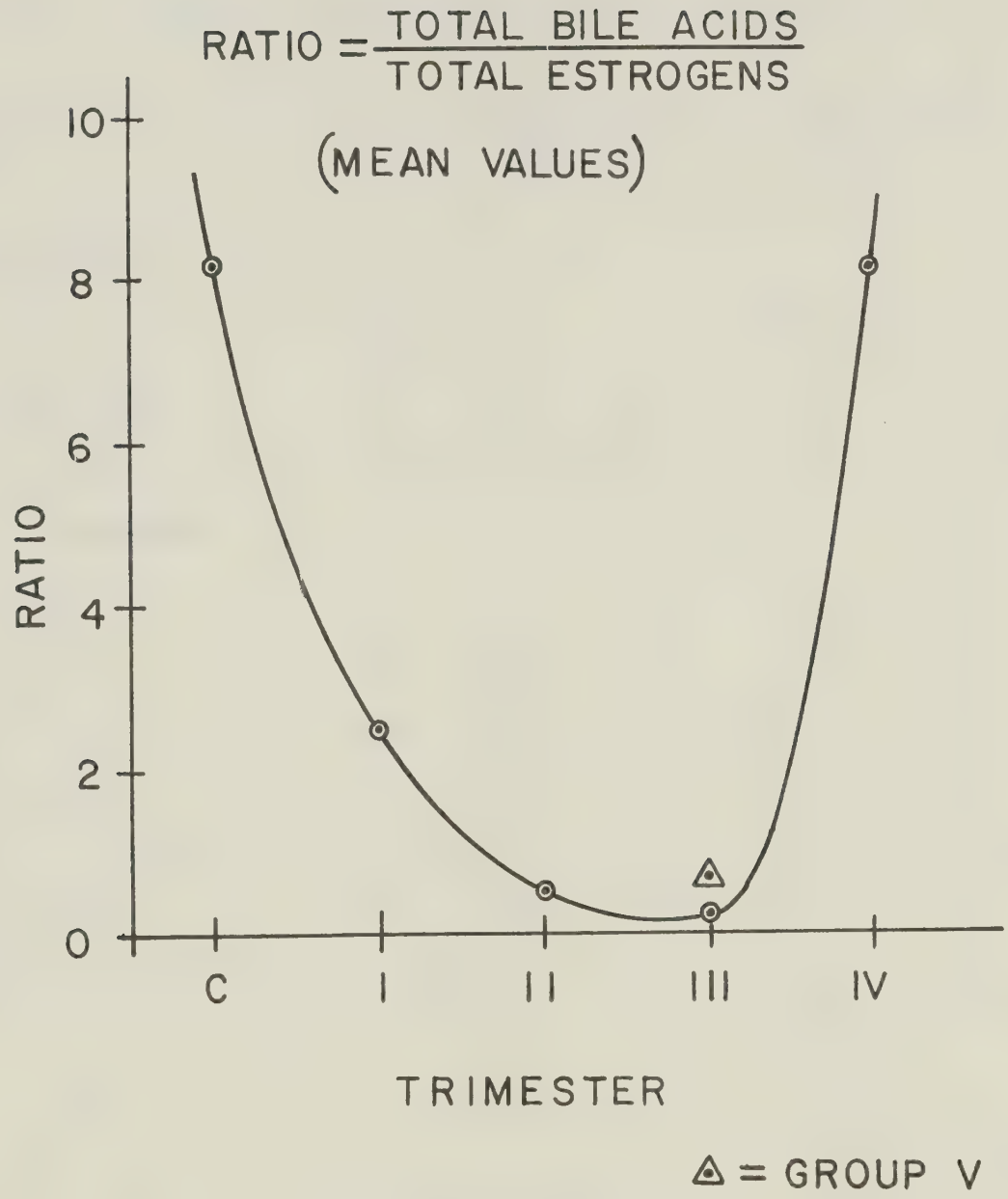

Figure 12. Ratio of Total Serum Bile Acids to Total Serum Unconjugated Estrogens.

Table 12. Ratios of Total Bile Acids to Total Unconjugated Estrogens

Group		n	Mean	SD	Range
Non-Pregnant					
Controls - C		33	8.1	6.4	1.6 - 28.0
On Oral - OC Contraceptives		17	19.3	12.9	1.4 - 46.0
Normal Pregnant					
Trimester	I	26	2.5	2.1	0.3 - 8.3
Trimester	II	26	0.5	0.6	0.1 - 3.7
Trimester	III	26	0.3	0.3	0.1 - 1.6
Post-Partum	IV	17	8.2	5.3	2.0 - 19.0
Cholestatic Pregnant	V	10	0.6	0.6	0.1 - 1.2

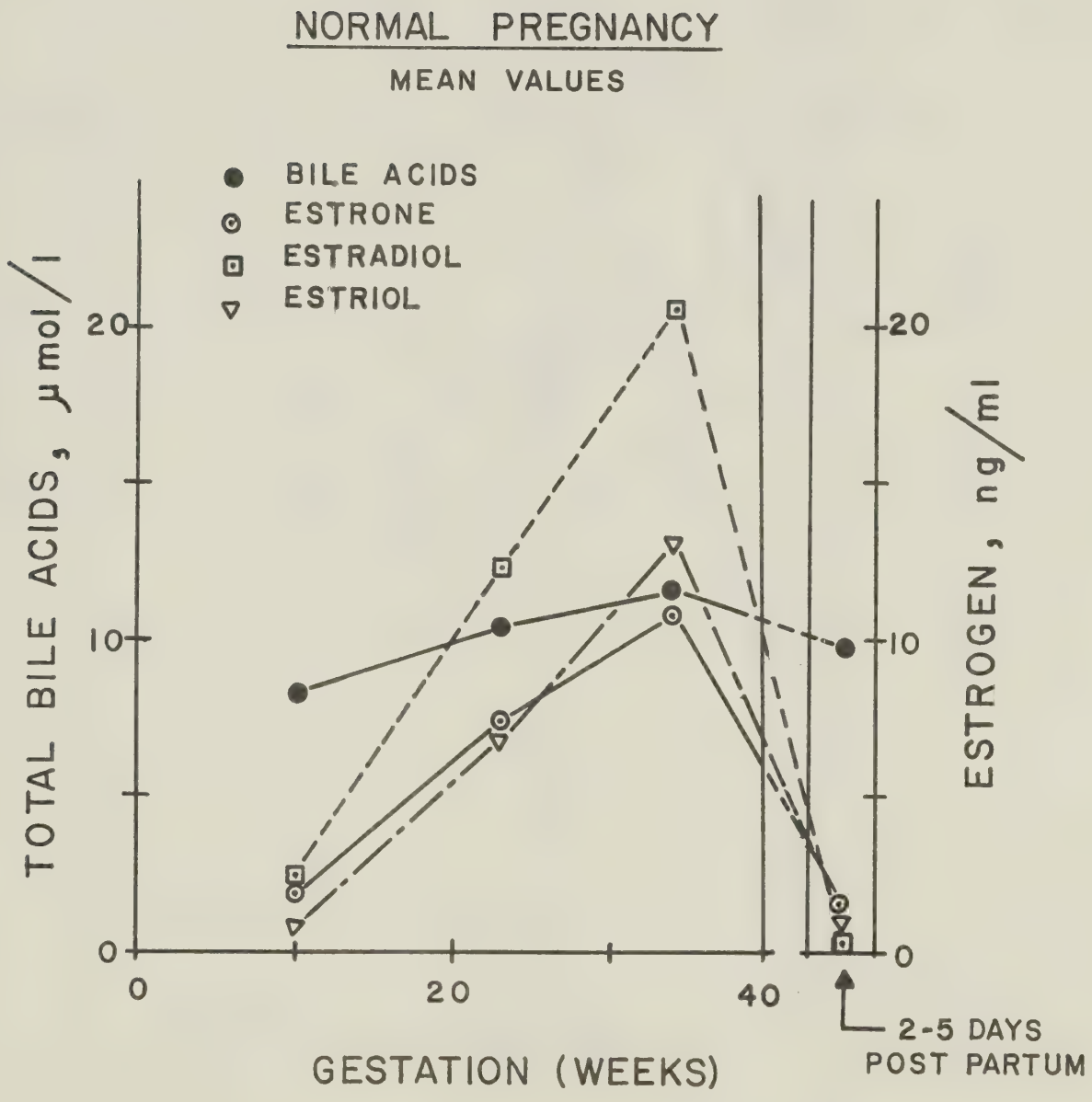

Figure 13. Mean Values for Serum Bile Acids, Estrone, Estradiol, Estriol in Normal Pregnancy.

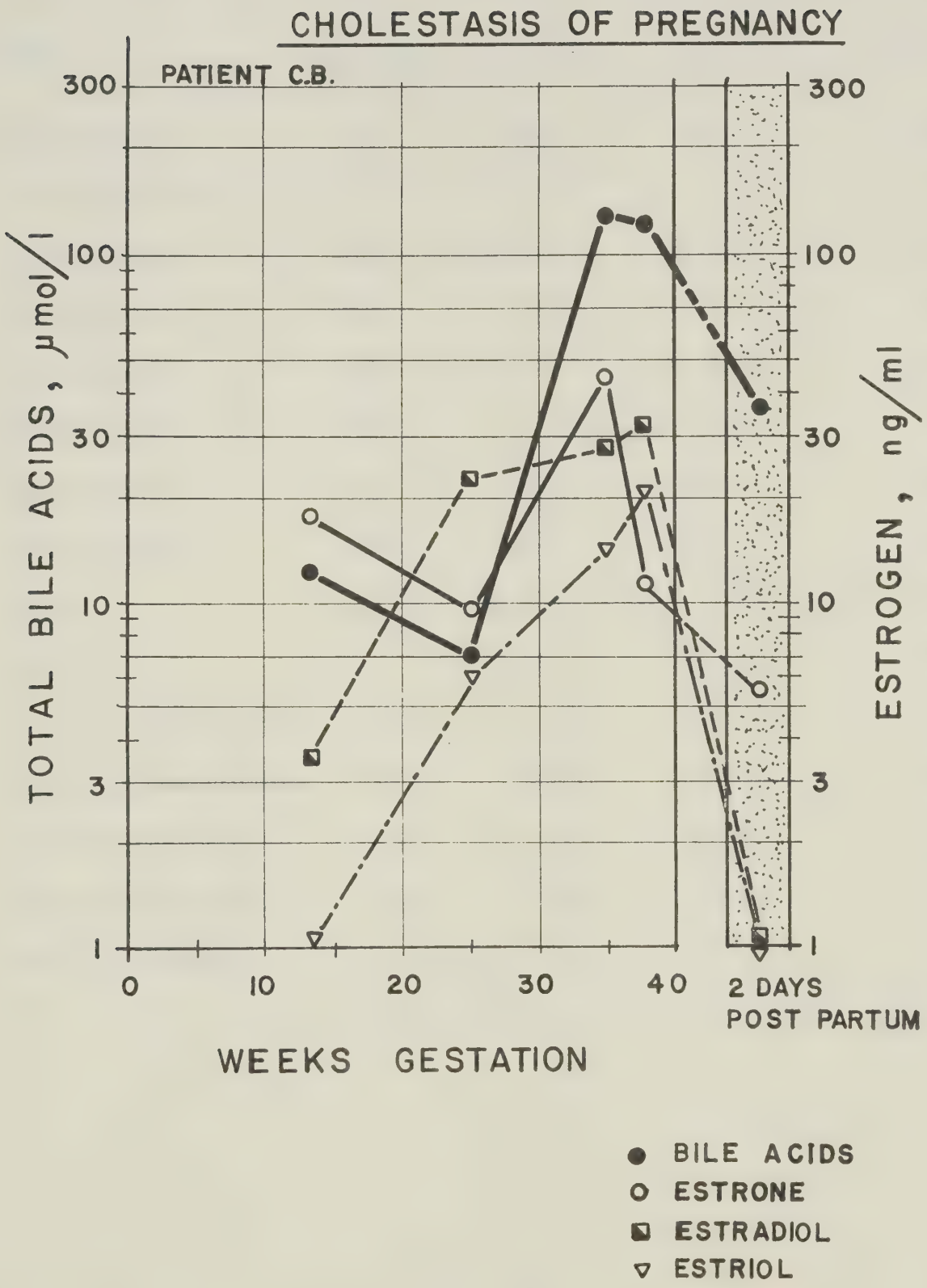

Figure 14. Recurrent Cholestasis of Pregnancy: Patient C.B.

Table 13. Recurrent Cholestatic Jaundice of Pregnancy

Patient C.B. - 21 years - Second Pregnancy Gestations

Test	13 wk	25 wk	35 wk	38 wk
ALP(IU/l)	91	143	468	466
T.BILI(mg/dl)	0.2	0.3	1.0	1.3
LDH(IU/l)	169	151	219	246
SGOT(IU/l)	31	22	47	81
SGPT(IU/l)	26	17	46	122
5'NT(IU/l)	10.1	6.6	50.2	23.7
TRIG(mg/dl)	163	325	492	530
CHOL(mg/dl)	176	320	540	358
LIPOS	N	Marked β and slow moving pre-β		
LP-X		Neg	Trace	Trace
T.Bile Acids(um/l)	12.2	7.0	133	125
T.Estrogens(ng/ml)	21.8	39.5	88.2	63.9
Estrone(ng/ml)	18.2	9.5	44.7	11.6
Estradiol(ng/ml)	3.6	23.4	28.9	31.3
Estriol(ng/ml)	0	6.6	14.6	21.0

Total estrogens at 13 weeks' were almost twice the normal, but were within the normal range of values during the second and third trimesters. The bile acids dropped during the second trimester despite a continuing rise of unconjugated estrogens.

F. Pruritus Gravidarum and Cholestasis

Tables 14 and 15 demonstrate the findings shown in cholestasis of pregnancy and pruritus gravidarum. Comparison of total bile acids and estrogens also demonstrate significant differences.

Table 14. Results of Liver Function Tests and Lipid Analyses for Group V Patients (Women in their Third Trimester with Cholestasis and/or Pruritus Gradivarum) and two Second Trimester Patients with Pruritus Gravidarum.

Patient	T.Bili mg/dl	ALP	5'NT IU/l	SGOT	SGPT	LDH	TRIG mg/dl	CHOL mg/dl
Group V - Third Trimester								
C.B. -	1.3	466	23.7	81	122	246	530	358
C.Z. -	2.6	442	42.7	100	86	158	354	354
S.B. -	2.0	583	32.2	51	60	174	338	276
J.B. -	1.3	350	24.4	250	240	258	360	239
J.T. -	1.3	221	15.0	339	374	187	296	224
S.M. -	1.3	212	18.4	90	32	144	354	336
G.A. -	-	-	37.0	-	17	-	418	248
D.T. -	0.3	134	11.1	23	10	157	235	233
L.M. -	0.7	78	11.4	20	43	168	218	247
J.L. -	0.6	238	15.4	24	10	120	550	358
Second Trimester								
R.G. -	0.3	52	4.4	32	17	192	132	236
A.T. -	0.2	45	10	11	6	139	186	170

LP-X Present in sera from patients C.B, S.B, J.B.

Table 15. Results of Total Bile Acids and Estrogens for Group V Patients (Women in their Third Trimester with Cholestasis and/or Pruritus Gravidarum), and two Second Trimester Patients with Pruritus Gravidarum.

Patient	Total Bile Acids	Total Estrogens	Ratio **	Estrone	Estradiol	Estriol
	umol/l	ng/ml		ng/ml	ng/ml	ng/ml
Group V - Third Trimester						
C.B.	125	63.9	1.9	11.6	31.3	21.0
C.Z.	31.6	47.5	0.7	28.4	11.8	7.3
S.B.	80.4	67.3	1.2	38.3	20.8	8.2
J.B.	96.4	80.0	1.2	42.8	16.6	20.6
J.T.*	19.2	84.3	0.2	56.0	15.8	12.5
S.M.	22.4	59.4	0.4	33.0	16.2	10.2
G.A.	2.0	27.8	0.1	12.1	6.5	9.2
D.T.	7.7	35.3	0.2	8.6	15.8	10.9
L.M.	10.2	38.5	0.3	16.4	14.3	7.8
J.L.	1.6	53.1	0.1	20.2	19.6	13.3
Second Trimester						
R.G.	4.6	58.1	0.1	17.6	27.4	13.1
A.T.	10.4	55.1	0.2	21.1	26.0	8.0

* J.T. - had cholecystostomy and T-tube drainage of common bile duct

** - Total bile acids/total estrogens.

CHAPTER V

DISCUSSION

Results from this study demonstrate that changes in the metabolism of bile acids and estrogens occur during pregnancy. These are exaggerated when pruritus and cholestasis supervene. Although there have been several reports of serum unconjugated estrogens throughout normal pregnancy,[163] [186] [187] [188] levels in cholestasis and pruritus gravidarum have not been determined, and the only reports of serum bile acid levels during normal and cholestatic pregnancy have been for patients near term.[93] [94] [189]

Non-fasting patients were sampled at the same time of day for each trimester, thereby serving as their own "controls", and eliminating variation in bile acid and estrogen levels.[190] Postprandial serum bile acids are considered by some[191] to be more meaningful than fasting levels because of enterohepatic stimulation after a meal.

Sample storage at -20° for long periods did not affect bile acid and estrogen levels, and liver enzymes were unaffected with the exception of alanine aminotransferase, which showed a 50% decrease.

The age range of patients for each of the groups was approximately the same with the exception of normal controls, as the use of oral contraceptives made it difficult to find normal controls who were not using the

"pill". Subjects for this group were a little older than those from other groups, therefore possibly disposing to slightly increased levels for some of the analyses as compared to younger women, and consequent wider variability in comparison of the control group to the test groups.

"Cholestatic" patients were categorized according to gestation instead of severity of cholestasis, allowing comparisons with third trimester normal pregnant patients. Pruritic patients who had increased alkaline phosphatase, 5' - nucleotidase, and serum bile acid levels above 20umol/l, were considered to be cholestatic. Serum bile acid levels seemed to be a more sensitive index of diminished bile flow than bilirubin and aminotransferase levels, in agreement with Sjovall,[93] and others.[192]

Total and fractionated serum bile acid concentrations are extremely low in the normal, ranging from 0.4umol/1 to 13.0umol/l using the enzymatic-fluorimetric technique, and from 0.6umol/1 to 4.2umol/l with gas-liquid chromatography (GLC). Total bile acid estimations in serum under the best of conditions are difficult because of interferences from neutral lipids and losses from protein adsorption. Gas chromatography, attempted during the initial stages of method development, was found to be more time consuming, requiring 5ml to 10ml of serum from normal pregnant patients, with lower recoveries than the enzymatic procedure (50% to 60%).

The enzymatic technique had an accuracy and sensitivity compatible with the finding of Schwarz,[182] but the coefficient of variation for the method was somewhat higher, (16.3% versus 2.0%). Careful control of the XAD-2 extraction procedure was an important determinant of accuracy, and fluorometry required strict control of environmental conditions to eliminate interferences. The problem of fluorescence quenching, probably caused by incomplete removal of neutral lipids by high-speed centrifugation and corrected only by use of a "serum standard", may explain the higher coefficient of variation obtained. Attempts to eliminate these interferences by millipore filtration, solvent extraction of dried XAD-2 extracts, and thin-layer chromatography, all proved unsatisfactory. Purified hydroxysteroid dehydrogenase was more effective in eliminating interferences, at low bile acid concentrations, than crude extracts of the enzyme.[193] Spectrophotometric measurement of NADH absorbance at 360nm was too insensitive for the low concentration of bile acids normally present in serum, therefore necessitating the use of fluorometry. The influence of C19 and C21 steroids containing 3α-hydroxyl groups was negligible since these steroids are conjugated to either sulfate or glucuronate, and concentrations of the free components are low, making their interference minimal.

More sensitive, precise, direct, faster procedures such as radioimmunoassay or high-pressure-liquid-chromatography,

would be preferred alternatives for future studies. Gas chromatographic fractionation of bile acids would be acceptable for sera containing increased concentrations.

Our normal range of control values (Group C) was similar to those reported by others[94, 182 194 195] and there was no significant difference in the recoveries of individual bile acids (Chapter III, F). Jaundiced "controls" had values varying from 1.6umol/1 to 178umol/1, (Table 7) comparable to a recent report of forty-eight patients with liver disease and levels up to 140umol/1, using the same enzymatic procedure.[196]

The method of choice for determining the three unconjugated estrogens was radioimmunoassay, because of specificity, sensitivity, and minimal preliminary separation of the estrogens prior to quantitation. Other procedures considered, involved GLC and fluorometry but were too insensitive and non-specific for first trimester and "control" estrogen measurements. Radioimmunoassay procedures did not have the desired sensitivity for samples from non-pregnant women, and 2.0ml volumes of serum were required to obtain precise analytical values. Lower recoveries and estrogen loss was accentuated probably by protein adsorption. Multiple extractions of 2.0ml samples improved the recovery, and sonication, which was not tested, might have helped alleviate the problem. In spite of this, recoveries were of the same order as reported by others.[186]

[187] [188] Corrections for the variability in serum estrogen levels in the groups studied, were performed and the corrected values correlated satisfactorily with those from the literature.[184] [185] [188] [197]

Specificity of estrogen antisera is reportedly high with that prepared from 6-carboxime-BSA conjugates, because conjugation at the C6 position does not structurally interfere with the functional groups of these steroids.[188] Antisera to estradiol-17β and estriol respectively, had high specificity, with negligible cross-reactivity from other steroids (Table 2). Estrone antiserum, however, was not as specific, cross-reacting substantially with estradiol-17β. Cross-reactivity determinations were performed for the three estrogen antisera and those for estrone were subsequently used to correct the assayed values. Further separation of estrone from estradiol on celite or LH-20 sephadex would have given a more accurate reflection of the interference. However, sample size restrictions due to the number of tests performed on each specimen, precluded this procedure. Despite this problem, values for estrone and the other unconjugated estrogens, during the three trimesters of pregnancy agreed with others[184] [185] [188] [197]

Lipoprotein-X, was found in sera from three patients with cholestasis of pregnancy, one of whom had a cholecystectomy several weeks after delivery. The "abnormal" lipoprotein was also present in sera from several jaundiced

"controls" who had high serum bile acid values and clinical extrahepatic cholestasis.

The women using oral contraceptives were all taking combination preparations containing a progestogen and either ethinyl-estradiol or mestranol. Although liver function values were within normal limits, the mean values for certain liver enzymes and triglycerides were slightly higher than normal (Table 3). Ovarol was associated with increased aspartate and alanine aminotransferase values for one woman in spite of normal serum total bile acids, possibly indicating early changes in hepatocytic endoplasmic reticulum involving stimulation of enzyme production and release, prior to evidence of cholestasis.[66] Raised values for aminotransferases and alkaline phosphatase in serum have been reported in a small percentage of women receiving ethinyl-estradiol or mestranol-containing preparations and in women who have developed jaundice while taking oral contraceptives.[12] The slightly decreased mean value for cholesterol in women on "the pill" and a tendency towards an increase of serum triglycerides is in accordance with others.[198] [199] This may be the result of increased microsomal triglyceride production and secretion, or decreased hepatocyte uptake from the circulation. Additionally, there may be an inhibitory effect on cholesterol production and secretion, or an increased hepatocytic uptake and/or secretion into the bile. Recent evidence shows that bile can become more saturated with

cholesterol in women using oral contraceptives than in controls,[21] and this may be attributed to decreased bile acid synthesis, especially of chenodeoxycholate, relative to cholesterol synthesis and excretion. On discontinuing therapy, the increased lithogenicity of the bile reverts to normal.

Results from our study showed no significant difference between mean values for total bile acids in controls and women taking oral contraceptives. The range and mean values for the latter group were slightly less, and with a larger number of samples, a subsequent statistical difference might have been shown, however, the age differences between the two groups of women might also account for this finding. Significant changes of the unconjugated estrogens in the group on oral contraceptives compared to controls, may reflect depressed estrogen synthesis caused by the effect of low dose synthetic estrogens and progestogens on the hypothalamic-pituitary axis.[200] The difference between estradiol mean values for both groups (Table 10) may represent increased conversion to estriol via estrone in women on the pill, due to increased stimulation of hydroxylation in the hepatocyte (Figure 6), with consequent increased secretion into the enterohepatic circulation. Depressed estradiol values account mainly for the drop in total estrogens when compared to control values, and is in keeping with others who have found that the ovaries show morphological changes and become inactive during oral

contraceptive therapy, with a marked decline in urinary output of estrogen and progesterone metabolites.[200] Estriol levels were shown to be the same as control levels, and could be explained by metabolism of synthetic estrogens to an estriol-like metabolite. The difference in mean ratio of total bile acids to total estrogens for the two groups (Table 12), may demonstrate a decrease of bile acid synthesis in women on the pill, and a further decrease of estrogen synthesis compared to normal controls.

The significant increase of serum bile acids and unconjugated estrogens seen during normal pregnancy, may reflect a gradual reduction in their secretion rate by the hepatocyte into the enterohepatic circulation, or a reduced uptake from the circulation. Supportive evidence may be derived from changes in serum triglycerides and to a lesser extent cholesterol seen in pregnancy and biliary stasis.[27 82] Cholestasis of pregnancy was associated with higher concentrations of bile acids and estrogens, with more overt changes in serum lipids and liver enzymes than pruritus gravidarum. Normal bile acid concentrations have been described by Sjovall[93] in patients with pruritus gravidarum prior to the onset of cholestasis, although he considered bile acid levels to be the most sensitive index of cholestasis. This is in agreement with reports of pruritus gravidarum as a milder form of cholestasis of pregnancy where the only difference is the degree of impairment of hepatic excretory function and patients remain anicteric

with no morphologic evidence of cholestasis on liver biopsy.[65 201]

Increased levels of serum bile acids in cholestasis of pregnancy, reportedly 10 to 100 times those of normal pregnancy,[93 94] are primarily due to marked increases of cholic and chenodeoxycholic acids. Sjovall,[93] using GLC, found that the respective serum ratios for the hydrolyzed components of cholic, chenodeoxycholic, deoxycholic acids, were 1.0:1.0:0.9 in normal pregnancy, and 4.5:1.0:0.1 in cholestasis. The serum bile acids were conjugated except for one patient in whom free bile acids were detected, and the ratios were similar to those found for patients with hepatobiliary disorders. These findings were confirmed by Makino.[94] Interruption of the enterohepatic circulation, with subsequently less cholate reaching the intestine for deoxycholate synthesis, and decreased hepatic secretion, might explain the relatively increased concentrations of deoxycholate which are low compared to cholate and chenodeoxycholate. Reduction of deoxycholate destroys the bile acid negative feedback control of cholic and chenodeoxycholic acid synthesis, which would therefore be expected to continue in the presence of cholestasis, as also would the synthesis of cholesterol.[134 139] There have been no reports of lithocholate in serum during pregnancy, although Pellizziari[202] did find trace amounts in sera from 20% of normal individuals studied. Combes,[87] and Mistilis,[63] in studies of BSP retention, using a prolonged infusion

technique, showed that the hepatic relative storage capacity was markedly increased during the last half of pregnancy. They associated these changes with a possible intrinsic change in the liver. These findings could be equated with the gradual rise of serum bile acids during pregnancy, which are accentuated in cholestasis. Simcock[89] suggested that rising levels of estrogens and progesterone and the increasing excretory load on the liver imposed by the metabolism of these steroids, is responsible for progressive impairment of BSP elimination and probably other substances.

Normal pregnancy reduces bile acid synthetic and secretory rates in rats,[23] and although the estrogens and oral contraceptives have been implicated in the reduction of bile acid synthetic rates and pool size,[21 203 204] the mechanism whereby these steroids may cause intrahepatic cholestasis has not been elucidated. Studies in which there is reduction of the BSP excretory rate in women receiving mestranol-norethynodrel preparations with normal hepatic storage and conjugation, indicate that two different mechanisms may be affected in pregnancy, of which only the excretory mechanism is affected by estrogens.[87 205] The postpartum decrease of serum bile acids, liver enzymes and lipids, found in this study, may be the result of removal of the estrogenic effect at delivery.

The majority of patients who demonstrated changes in liver function tests during pregnancy, demonstrated

associated changes in serum bile acids. The increase of serum alkaline phosphatase activity during normal pregnancy has been attributed to heat-stable placental isoenzyme.[83] Normally, hepatic isoenzyme activity is localized on the canalicular membrane.[206] In cholestasis, there is a markedly generalized increase in alkaline phosphatase activity throughout the liver lobule, which has been interpreted as excessive excretory stimulation of the hepatocyte related to increased local concentration of bile acids,[207] and may be the reason for high alkaline phosphatase activity seen in cholestasis of pregnancy. High doses of estrogens have also been shown to cause increased alkaline phosphatase activity in rats.[206] 5'-Nucleotidase is located in the canaliculi and sinusoidal liver cell membranes,[207] whereas the aminotransferases are derived from hepatocytic cytoplasm and mitochondria.[208]

The disturbance in lipid metabolism associated with pregnancy may be secondary to stimulation of the microsomal endoplasmic reticulum, with possible impairment of hepatic uptake and excretion due to decreased lipoprotein lipase activity, or there may simply be increased fat mobilization.[91] Several workers have incriminated estrogens as a possible cause for the triglyceride and cholesterol increases which occur during pregnancy.[91 167] Svanborg[91] suggested that hyperlipemia may be responsible for pruritus, which may obtain support from the patients in our study who had pruritus with no evidence of cholestasis, but high

triglyceride values. However, their estrogen levels were more remarkable, which may favor a steroidal cause of pruritus.

When estrogens and their derivatives are considered, estrone is greatly increased in pregnancy due mainly to estrone sulfate. Conjugated estriol is high, whilst estradiol exists almost entirely in its "free" or unconjugated form and is thought to be mainly responsible for the estrogenic effect during pregnancy.[157] Loriaux[184] found unconjugated estradiol to have the highest concentration in plasma, which was twice that of estrone and four times that of estriol, and our results were in close agreement. He also reported twice as much estrone sulfate as estradiol, and concluded that the increasing load of estrogens, particularly estradiol, may reflect overloading of the dehydroxylating enzymes which convert estradiol to estrone, or an enzyme further along the pathway in the production of estriol, Figure 6. Conjugating enzymes may also be affected, giving rise to the accumulation of unconjugated estrone and estriol. Estrogens are the only steroid hormones with a large enterohepatic circulation,[159] and impaired excretion of their conjugates may affect hepatic enzymes involved in their metabolism and biological inactivation, causing an increase of unconjugated estrogens. Both estrone and estradiol are considered to affect liver enzymes,[62 88] and their accumulation might predispose to the mild cholestatic effect in normal pregnancy, demonstrated in

the present investigation. Biliary estrone is mainly sulfated whereas estradiol and estriol occur as glucuronates[155] and consequently urinary excretion of bile acid[48] and estrogen sulfates[94] may be increased in obstructive jaundice due to interruption of the enterohepatic circulation.

The variation of estrone in patients with cholestasis and pruritus gravidarum, would lend support to the hypothesis of a further decrease of dehydroxylation or defective production of estriol. Similarly, the marked increase in estrone when compared to normal pregnancy, strongly suggests defective conversion of estrone to its sulfate, or other metabolites, since estriol levels remain essentially identical to those of normal pregnancy. This might lead to enhanced sulfation and urinary excretion of estradiol and estriol, the slightly lower estradiol values resulting from enhanced conversion of estradiol to estriol by an alternate pathway. Supportive evidence by Adlercreutz,[155] showed that bile from non pregnant patients with jaundice and cholelithiasis had increased estriol sulfate, and little or no estriol glucuronates. Later work[95] showed that urinary estriol-16-glucuronate and estrio-3-sulfate-16-glucuronate were increased, whereas estriol-3-glucuronate was reduced in cholestatic jaundice of pregnancy. Total urinary estriol excretion was the same as in normal pregnancy and it seemed that conjugation with glucuronic acid in the 16 or 17 position was more extensive,

with a simultaneous decrease of estriol conjugated in the C3 position and as the double conjugate 3-sulfate-16-glucuronate. Estriol-3-glucuronate synthesis is intestinal,[155] and consequently, due to the decreased estrogen enterohepatic circulation in cholestasis, reduced levels of C3 conjugated products would be anticipated. In a more recent report,[209] Adlercreutz showed that the relative urinary excretion of 16-epiestriol and estriol were depressed and their immediate precursors, 16α-hydroxyestrone and 16β-hydroxyestrone respectively, were increased in cholestasis of pregnancy and pruritus gravidarum. There was a uniform decrease in concentration of all biliary estrogens when measured in some of these patients, and estriol excretion was more severely impaired than estrone. Alternatively, Sjovall and Sjovall,[210] studied plasma steroid sulfates in 17 women with pruritus and cholestasis of pregnancy and found six steroids not normally present. Absolute and relative concentrations of steroid sulfates were strikingly different from normal, the main changes being increased concentrations of C21 steroids with 3α,5α and 3α, 5β configurations. They concluded that these changes were secondary to impaired biliary excretion and disturbed steroid metabolism.

In this study, relative concentrations of each of the unconjugated estrogens remained constant throughout normal pregnancy. Disruption of this balance, demonstrated in cholestasis of pregnancy, indicates an alteration of estrone

and estradiol metabolism, Figure 11. The maintenance of bile acid to total estrogen ratios seen in normal and cholestatic patients, despite marked differences in concentrations between both groups (Figure 12), may indicate similar hepatic secretory mechanisms for bile acids and estrogens.

Watanabe[211] recently showed that the ratio of cholate to chenodeoxycholate in bile was diminished by estrogen in cholestyramine-treated rats. Low doses of esradiol-17α, reportedly stimulated chenodeoxycholate excretory rates, whereas high doses reduced cholic acid levels. An intermediate dose showed both factors to be contributory to the decreased ratio, and prolonged treatment produced a reduced excretory rate of both bile acids in cholestyramine-treated and untreated animals. Although cholestyramine interruption of the enterohepatic circulation provided information concerning the effect of estrogens on primary bile acids, consideration of the effect of such an interruption on the deoxycholate negative feedback control of chenodeoxycholate synthesis, was not discussed. Despite this criticism, Watanabe's findings compared to ours, indicate that perhaps the "low dose estrogenic effect" was being observed by us in women using oral contraceptives, whereas the "high dose effect" was seen in pregnancy and associated cholestasis. If concentrations of serum bile acids are considered to be an index of bile acid secretion,[192] the decreasing ratio of total serum bile acids to total serum estrogens, during normal pregnancy (Figure

12), could imply that hepatic hydroxylating and conjugating mechanisms become overloaded as a result of the increased estrogen production during pregnancy, with consequent impairment of bile acid secretion.

It is suggested that concentrations of estrogens during pregnancy may have a competitive inhibitory effect on microsomal enzymes involved in bile acid hydroxylation and conjugation, with a consequent disturbance in hydroxylation, which may be the initiating step in cholestasis, interfering with micelle formation, disturbed bile salt secretion, and bile stasis in the liver. An accumulation of bile acids, particularly dihydroxy or monohydroxy derivatives, would inhibit microsomal mixed-function oxidases further by competitive inhibition, their detergent property resulting in a hypoactive endoplasmic reticulum and eventual hepatocellular necrosis.[31] Protective conjugative mechanisms including sulfation and glucuronidation are inadequate to remove hepatotoxic bile acids during severe cholestasis. Estrogens would also accumulate in the liver as a result of impaired bilary secretion, exerting an even greater effect on hepatocyte metabolism. Enhanced sulfation of estrogens and bile acids probably occurs, but was not investigated in this study. Consequently as the disturbed metabolism of bile acids and estrogens result in cholestasis, impairment of the enterohepatic circulation during pregnancy would cause decreased negative feedback control of cholesterol and bile acid synthesis via 7α-hydroxylase and HMG-CoA reductase.

Bile acid synthesis and secretion would remain depressed due to the already present estrogenic effect. Another factor which might lead to reduced bile acid secretion is the increased capacity for intestinal bile acid reabsorption during pregnancy, which may cause an increased rate of pool "cycling", resulting in decreased synthesis and pool size and subsequently reduced secretion.[212] A diminished bile acid pool precedes the formation of cholesterol gallstones in man.[57]

Attention has already been focused on the relationship between normal pregnancy and the exaggerated response with biliary stasis seen in pregnancy cholestasis. This abnormal response may be related to an inherent enzyme defect in the liver cell, whereby large doses of estrogens are not metabolized as efficiently as in the normal. This was demonstrated in patient C.B. at 13 weeks' gestation who had an abnormal unconjugated estrone level, with a normal gestational total bile acid concentration. The second trimester drop of estrone for patient C.B., may indicate a stimulated conversion to estriol; however, the increased level of unconjugated estradiol may be accounted for, by an inhibition of 17β hydroxydehydrogenase (Figure 6). The two second trimester patients with pruritus gravidarum (Table 15) had similar elevations of estradiol. Severe itching in these patients is strongly suggestive of an estrogenic cause, or "factors" other than bile acids.[213] Cholestyramine, the enteric, non-absorbable, anion exchange

resin relieves itching by sequestering bile acids,[214] and possibly estrogens[72] during their enterohepatic circulation, presumably lowering serum and tissue levels. Sjovall[210] found changes in plasma steroid sulfates which preceded elevations of serum bile acids in pruritus gravidarum, and also found that cholestyramine improved, but did not eliminate the pruritus. The fact that patient C.B.'s bile acids dropped at 25 weeks', despite a continued rise of unconjugated estrogens, may reflect either decreased synthesis or increased excretion. Decreased bile acid synthesis may have resulted in cholestasis during the third trimester with consequent elevation of serum bile acids. Total estrogen production in cholestasis of pregnancy does not appear increased in individual patients (Table 15) despite an increased mean value for the group when compared to normal third trimester pregnancy, Table 8. A wide range of overlap for the two groups was shown.

A further postulate to support the increasing concentrations of free bile acids, may involve the sulfating enzymes, which could be competitively inhibited by the large increase in estrogen during pregnancy. This, in particular, could lead to an increase in free lithocholate and chenodeoxycholate, the former having a characteristic hepatotoxic action.[29] Most bile acid hydroxylation reactions are mediated by the non-specific microsomal P450 dependent drug metabolising enzymes, and since interaction between drugs in competition for this microsomal detoxifying

function have been demonstrated,[215] a similar interference may occur between estrogens and bile acids, resulting in a disturbed ratio of primary bile acid levels. Methyltestosterone competitive inhibition of taurodeoxycholate metabolism is strongly suggestive of drug impairment of bile acid metabolism with possible elevations of hepatotoxic bile acids.[31] Evidence of reduced 12α - hydroxylation in rats during experimental cholestasis following bile duct ligation, and drug interaction with hepatocellular membrane function has been presented by Greim.[31]

Estradiol inhibits rat liver parenchymal cell 6β-hydroxylation of cortisol to 6β-hydroxycortisol and β-cortol,[216] and the conversion of cholate to chenodeoxycholate.[173] The enzyme 6β-hyroxylase, is known to be important in the rat for its detoxification of chenodeoxycholate to β-muricholic acid.[38] Although confirmation is lacking in man, one cannot rule out this latter pathway as an alternate means of chenodeoxycholate detoxification when sulfation and glucuronidation are impaired. The increase of serum cholate demonstrated in cholestasis[93] may result from the detoxification of chenodeoxycholate to the less toxic cholate. Reference has been made previously to the structural and functional changes reported in the smooth endoplasmic reticulum of the liver, due to cholestasis.[41] Chenodeoxycholate causes a decrease in 6β-hydroxylase activity and P450 level in

rats,[217] and the Cytochrome P450 dependent reactions are believed to prevent accumulation of detergent dihydroxy bile acids in rats. Taurocholic, taurodeoxycholic, and taurochenodeoxycholic acids fed to rats inhibit both 7α and 12α-hydroxylases,[218] as do cholic and chenodeoxycholic acids.[136]

Conversion of estrone to methoxyestrone, hydroxyestrone, estriol and estrone sulfate are decreased in cirrhosis, and a decrease in 2α-hydroxylation and impaired reduction of 16α-hydroxyestrone have been reported in cholestasis and cirrhosis.[209] [219] This disturbance in conversion may also be caused by interruption of the enterohepatic circulation. Administration of estrogens to patients with liver disease has resulted in increased amounts of urinary unconjugated estrogens, explained by impaired hepatocellular uptake and possible reduction of glucuronyl transferases in the hepatocyte.[160]

Czygan[220] recently isolated human liver microsomes from patients with and without gallstones, and found that stone carriers had reduced 16β-hydroxylation of taurolithocholate compared to non-carriers. However, both groups had a normal cytochrome P450 content, and since 16β-hydroxylation is cytochrome P450 dependent, it was assumed that a selective defect in cytochrome P450 dependent biotransformation may play a role in gallstone formation. Perhaps in pregnancy, there is competitive inhibition of 16β-hydroxylation of bile

acids by estrogens, thus producing the same effect. If 12α-hydroxylase activity were depressed, side chain cleavage would precede 12α-hydroxylation, leading to the formation of monohydroxy and dihydroxy bile acids which would then be hydroxylated in the 6 and 7 positions. An enzyme system capable of hydroxylating taurolithocholate to taurohyodeoxycholate has been demonstrated in human liver microsomes.[221] Alteration of smooth endoplasmic reticulum is the primary event in cholestasis development, and this is the region of the hepatocyte where bile acid and estrogen hydroxylations occur.[33]

Further investigations should include measurement of bile acid and estrogen hydroxylating and conjugating enzymes in normal and cholestatic pregnancy, in an effort to determine those affected. Animal studies demonstrating the effect of various estrogens on bile acid hydroxylation are also indicated, with the measurement of individual serum bile acids and estrogens, their intermediates, and their sulfated and glucuronidated conjugates.

CHAPTER VI

CONCLUSIONS

The following conclusions were made from this study.

1. The metabolism of estrogens and bile acids is markedly affected by pregnancy, becoming increasingly apparent as pregnancy progresses. These changes are more pronounced with the clinical onset of pruritus and cholestasis.

2. Serum unconjugated estrogens increase at a faster rate than bile acids, implying that bile acid levels increase during pregnancy in response to previously increased estrogen levels, resulting from reduced synthesis and secretion.

3. The jaundice and cholestasis associated with pregnancy and oral contraceptive therapy probably has a genetic basis. This may result in a sensitivity to the levels of estrogens associated with normal pregnancy, leading to an inability of the liver cell to adequately metabolize the increased estrogen load, with subsequent impairment of hepatic excretory function.

4. There appears to be a balance between the unconjugated estrogens where relatively constant amounts are maintained throughout normal pregnancy, with an ordered dominance of estradiol, estriol, and estrone. In cholestasis, however, this balance is disrupted, and estrone becomes dominant, followed by estradiol then estriol.

5. The postpartum drop of serum bile acids in normal pregnancy, may be the result of removal of the "estrogenic"

effect.

6. Pregnancy is cholestatic. Slight impairment of the enterohepatic circulation, which may be caused by an estrogenic effect on hydroxylating and conjugating enzymes for bile acids and estrogens, may result in the lack of negative feedback control of bile acid synthesis. However, such a hepatotoxic effect of estrogens may prevent enzyme response, with resultant decreased bile acid synthesis. There may be competition between estrogens and bile acids for hydroxylating enzymes, and disturbed hydroxylation may interfere with micelle formation, initiating reduced secretion, biliary stasis, and possible cholelithiasis. A build-up of hepatotoxic bile acids would inhibit microsomal hydroxylations even further, perptuating cholestasis.

7. Bile acids and estrogens have similar secretory mechanisms, as indicated by the similar ratios of total bile acids to total estrogens in cholestasis of pregnancy as in normal pregnancy, despite the higher concentrations of both parameters in the former group.

8. One can postulate that estrone and possibly estradiol, may be responsible for initially altering hepatocytic smooth endoplasmic reticulum, affecting triglyceride, bile acid, and estrogen metabolism, at the hydroxylase and possibly P450 level. This would result in disturbed bile acid secretion, increased bile lithogenicity, and secondary damage to the endoplasmic reticulum due to the cholestasis, causing a further reduction of enzyme activity.

9. "Itching" seen in pruritus gravidarum may be caused by estrogens or lipids initially, when serum bile acids are normal, the bile acids becoming contributory as cholestasis develops and bile acid levels increase.

10. Metabolic changes in estrogens also occur in women using oral contraceptives. Serum bile acids, however, are essentially normal, with possible slight impairment of production. The slightly lower estrone and estradiol levels compared to controls, were seen as a secondary response to the effect of low-dose estrogens on the hypothalamic-pituitary axis.

11. We did not show whether metabolic or solely secretory changes of bile acids occurred during pregnancy. Further work involving bile acid fractionation is required to elucidate this.

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